WATER RELATIONS OF FUNGI
WITH PARTICULAR REFERENCE TO XEROPHYTIC SPECIES

by

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STATEMENT OF ORIGINALITY

Except where acknowledged, this dissertation is my own work.

Elizabeth J. Lund.

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Chapter 2 is in the form in which it has been accepted for publication in Transactions of the British Mycological Society.

ABSTRACT

Radial growth rates and hyphal osmotic potentials have been measured in eight species of fungi representing a wide range of tolerances to water stress. External water potential was controlled by the addition of KCl or sugar (sucrose or glucose); colonies were separated from the solid media by cellophane. The drought sensitive species had higher growth rates and sharper optima than did the xerophytes. Large positive turgor potentials were maintained in all species even when the external potential severely inhibited growth. A tendency for turgor to increase at low water potentials, both within and between species, was observed. Age and position of the hyphae affected turgor.

The ethanol soluble carbohydrates, amino acids and principal ions of three of these species were analysed. The principal organic solutes to accumulate in response to both steady state stress and osmotic shock were glycerol in Chrysosporium fastidium and Penicillium chrysogenum and proline in Phytophthora cinnamomi. The osmoticum used in the growth medium, whether KCl or sugar, was also a significant component of the internal osmotic potential. The polyols mannitol, arabitol and erythritol were also present in the first two species but arabitol only in small amounts in the last. Potassium was the predominant cation in all three species but there was insufficient chloride to maintain electroneutrality. The $K^+:Na^+$ ratio was related to the growth rate in P.cinnamomi and P.chrysogenum but increased linearly as the potential decreased in C.fastidium due both to accumulation of K^+ and exclusion of Na^+.

Higher contents of glycerol and the osmoticum were observed at the margin of P.chrysogenum than in older parts of the colony; the $K^+:Na^+$ ratio was also greatest at the margin.

Proline also accumulated at low water potential in three other species of lower fungi, Mucor hiemalis, Pythium debaryanum and a water mould (Saprolegniaceae). Glycerol was not found in any of the lower

fungi.

Some estimates of hyphal concentrations were made and these to some extent accounted for the measured hyphal osmotic potentials.

Respiration of the same three species was measured with an electrolytic respirometer at different water potentials, both steady state and shock. Low water potential decreased the hourly respiration rate of whole colonies, both of the same age and of the same radius, in proportion to the growth rate. The colony rate also decreased with time in some cases. A model of colony respiration rate proportional to the growing zone (circumference) plus maintenance of the non-growing zone (area) was proposed. An experiment to test this demonstrated that the unit respiration rate was ten times greater at the margin than in the remainder of the colony, but the latter was not insignificant. A logistic model to describe colony respiration was also considered.

Catabolite repression of the specific respiration rate by glucose and sucrose was most noticeable in P.cinnamomi and least in C.fastidium. Low KCl potentials enhanced the specific respiration rate.

Both hyperosmotic and hypoosmotic shock usually enhanced the colony respiration rate.

The NADP specific isocitrate dehydrogenases isolated from P.cinnamomi grown on different potentials and osmotica, showed the same activity.

The NADP isocitrate dehydrogenase activity from the three species was compared at different potentials and with different solutes. Proline and glycerol were the least inhibitory and KCl the most. Glycerol was able to relieve catabolite repression by glucose but not inhibition by KCl.

Light and electron microscope studies of the hyphae of the three species grown at different potentials were made. Low water potential increased wall thickness only in P.cinnamomi. High water potential slightly increased both wall thickness and cell size in C.fastidium. An

attempt to examine the effect of potential on the organisation of the hyphal apex was not successful. Various membrane structures were observed; membrane whorls in P.chrysogenum and C.fastidium, lomasomes in P.cinnamomi and structures resembling bacterial mesosomes in C.fastidium. It is possible that growth at low water potential results in a proliferation of membranes.

Glycogen granules were identified in P.chrysogenum and C.fastidium but not in P.cinnamomi.

The length of the apical cell of P.chrysogenum was related to the growth rate, but low water potential decreased the number of branches per apical cell. Water potential had little effect on morphology of C.fastidium but added fructose increased the length of the unbranched apex and decreased the number of branches per unit length.

Osmotic shock stopped growth. The time taken for recovery was a function of both the potential difference of the shock and of the growth rate of the species. Tips which burst did not recover and regrowth took place some distance behind the margin.

It was concluded that there were more similarities between the obligately xerophytic species (C.fastidium) and the facultative species (P.chrysogenum) than of either with the non-tolerant species (P.cinnamomi) in terms of their water relations. However the inability of C.fastidium to grow at low water potentials produced with salt was noteworthy and distinguished it from the other two species. It was tentatively suggested that a difference in membrane characteristics might be responsible.

CONTENTS

TITLE i

STATEMENT OF ORIGINALITY ii

ACKNOWLEDGEMENTS iii

ABSTRACT iv

TABLE OF CONTENTS vii

LIST OF FIGURES x

LIST OF TABLES xiii

LIST OF PLATES xvii

1. GENERAL INTRODUCTION 1

THE TERMINOLOGY OF WATER RELATIONS 1

WATER RELATIONS AND FUNGAL ECOLOGY 5

2. THE EFFECT OF WATER POTENTIAL ON FUNGAL GROWTH AND TURGOR 8

INTRODUCTION 8

MATERIALS AND METHODS 10

RESULTS AND DISCUSSION 16

3. THE EFFECT OF WATER POTENTIAL ON HYPHAL SOLUTES 26

INTRODUCTION 26

MATERIALS AND METHODS 28

THE EFFECT OF STEADY STATE OSMOTIC STRESS ON HYPHAL SOLUTES 33

RESULTS 33

DISCUSSION 49

THE EFFECT OF OSMOTIC SHOCK ON HYPHAL SOLUTES 63

RESULTS 64

DISCUSSION 79

THE EFFECT OF POSITION IN THE COLONY ON HYPHAL SOLUTES IN PENICILLIUM CHRYSOGENUM 82

RESULTS 82

DISCUSSION 86

THE EFFECT OF WATER POTENTIAL ON THE ACCUMULATION OF ORGANIC SOLUTES IN THREE LOWER FUNGI 89

RESULTS 89

DISCUSSION 91
GENERAL DISCUSSION 93

4. THE EFFECT OF WATER POTENTIAL ON RESPIRATION AND ACTIVITY OF A RESPIRATORY ENZYME 102
INTRODUCTION 102
MATERIALS AND METHODS 104
THE EFFECT OF STEADY STATE OSMOTIC STRESS ON RESPIRATION 111
RESULTS 111
DISCUSSION 129
THE EFFECT OF OSMOTIC SHOCK ON RESPIRATION 134
RESULTS 134
DISCUSSION 137
THE EFFECT OF GROWTH CONDITIONS OF PHYTOPHTHORA CINNAMOMI ON ISOCITRATE DEHYDROGENASE ACTIVITY 138
RESULTS 138
DISCUSSION 140
THE EFFECT OF WATER POTENTIAL AND SOLUTES ON ISOCITRATE DEHYDROGENASE ACTIVITY 141
RESULTS 141
DISCUSSION 146

5. THE EFFECT OF WATER POTENTIAL ON HYPHAL GROWTH, MORPHOLOGY AND ULTRASTRUCTURE 152
INTRODUCTION 152
MATERIALS AND METHODS 153
THE EFFECT OF STEADY STATE OSMOTIC STRESS ON ULTRASTRUCTURE 155
RESULTS 155
DISCUSSION 169
THE EFFECT OF STEADY STATE STRESS ON HYPHAL MORPHOLOGY 172
RESULTS 173
DISCUSSION 180
THE EFFECT OF OSMOTIC SHOCK ON GROWTH AND MORPHOLOGY 182
RESULTS 182
DISCUSSION 186

6. GENERAL DISCUSSION AND CONCLUSIONS 191

REFERENCES 200

APPENDIX 1. LIST OF SPECIES NAMES WITH AUTHORS MENTIONED IN THE TEXT 229

APPENDIX 2. OSMOTIC POTENTIALS OF MANNITOL SOLUTIONS 232

LIST OF FIGURES

2.1 Calibration of low water potential system with lithium chloride solutions and experimental values for glucose solutions of the same theoretical potential. 14

2.2 Effect of water potential on radial growth rates and turgor potentials of fungi grown on solid media adjusted with sugar and KCl. 23

3.1 Effect of water potential on the fresh weight to dry weight ratios and hyphal osmotic potentials of colonies (20 mm radius) of Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium grown on sugar osmotica and KCl. 35

3.2 Effect of water potential on the potassium to sodium ratios and the radial growth rates of Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium grown on sugar osmotica. 39

3.3 Effect of water potential on the potassium, sodium and chloride ion contents of Phytophthora cinnamomi and Penicillium chrysogenum grown on KCl. 40

3.4 Effect of water potential on the principal organic solutes in Penicillium chrysogenum and Chrysosporium fastidium. 43

3.5 Effect of water potential on the content of higher polyols in Penicillium chrysogenum grown on glucose and on KCl and on Chrysosporium fastidium grown on glucose. 44

3.6 Effect of water potential on the proline content of 47

	Phytophthora cinnamomi grown on sucrose osmoticum and on KCl osmoticum.	
3.7	The Embden-Meyerhof-Parnas scheme of glycolysis.	54
3.8	Principal pathways of polyol biosynthesis in fungi.	57
3.9	Effect of osmotic shock on the hyphal osmotic potentials of Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium.	66
3.10	Effect of hyperosmotic shock and hypoosmotic shock on the ion content of Phytophthora cinnamomi and after isoosmotic transfer of Chrysosporium fastidium to KCl.	70
3.11	Effect of hypoosmotic shock on the principal soluble carbohydrate content in Penicillium chrysogenum and Chrysosporium fastidium.	74
3.12	Effect of hyperosmotic shock on the principal soluble carbohydrate of Penicillium chrysogenum and of isoosmotic transfer to KCl on Chrysosporium fastidium.	75
3.13	Effect of hyperosmotic shock and hypoosmotic shock on the proline content of Phytophthora cinnamomi.	77
4.1	Effect of water potential on steady state growth and respiration rates in Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium grown on sugar.	113
4.2	Effect of water potential on steady state growth and respiration rates in Phytophthora cinnamomi and Penicillium chrysogenum grown on KCl.	114
4.3	Change in respiration rate with colony age in	116

Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium grown at the highest and lowest potentials tested and that which produced the maximum growth rate.

4.4 Change in calculated maintenance, growth zone and total respiration rates and observed total respiration rate with time in Penicillium chrysogenum grown on KCl osmotica. 123

4.5 Change in specific respiration rate and in the circumference to area ratio with time in Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium. 128

4.6 Effect of hyperosmotic shock and hypoosmotic shock on the colony respiration rate of Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium. 136

4.7 Effect of water potential on NADP isocitrate dehydrogenase activity from Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium assayed with glycerol, KCl and sucrose or glucose. 145

LIST OF TABLES

2.1 Effect of cellophane on radial growth rate. 16

2.2 Maximum daily growth rates. 17

2.3 Average turgor potentials and standard deviations and the range of potentials tested. 20

2.4 Comparison of hyphal osmotic potentials determined by psychrometry and tip bursting. 25

3.1 The ion content of Phytophthora cinnamomi grown at different osmotic potentials produced by the addition of sucrose. 37

3.2 The ion content of Penicillium chrysogenum grown at different osmotic potentials produced by the addition of glucose. 38

3.3 The ion content of Chrysosporium fastidium grown at different potentials produced by the addition of glucose. 38

3.4 The effect of water potential on the ethanol soluble carbohydrate content of Phytophthora cinnamomi. 45

3.5 The total amino acid pools extracted from hyphae grown on media of different water potentials. 46

3.6 The composition of the amino acid pools extracted from hyphae exhibiting maximum observed radial growth rates. 48

3.7 The fresh weight to dry weight ratios of Phytophthora cinnamomi, Penicillium chrysogenum and 65

Chrysosporium fastidium following osmotic shock.

3.8 The effect of hypoosmotic shock on the ion contents of Penicillium chrysogenum and Chrysosporium fastidium. 68

3.9 The effect of hyperosmotic shock on the ion content of Penicillium chrysogenum. 69

3.10 The effect of shock treatment on the minor soluble carbohydrate content of Penicillium chrysogenum and Chrysosporium fastidium. 73

3.11 The effect of hyperosmotic shock and hypoosmotic shock on the levels of the principal components and the total amino acid pool in Penicillium chrysogenum. 78

3.12 The ethanol soluble carbohydrate content of hyphae from different positions in colonies of Penicillium chrysogenum grown at three different osmotic potentials. 85

3.13 The effect of water potential on the organic solutes of three lower fungi. 90

3.14 Estimated concentrations of some hyphal solutes in P.cinnamomi, P.chrysogenum and C.fastidium. 98

4.1 The effect of the osmotic potential of the extraction buffer on the isocitrate dehydrogenase activity of Penicillium chrysogenum. 109

4.2 The linear correlation coefficients between colony respiration rate (colonies of equal radius) and two measures of growth rate. 112

4.3 The effect of water potential produced by the 118

addition of KCl on the width of the peripheral growth zone in Penicillium chrysogenum.

4.4 Respiration of the margin and interior of 9 day old Penicillium chrysogenum colonies of radius 26 mm grown on -2.5 MPa KCl. 119

4.5 Coefficients for the equation $dQ/dt = aR + bR^2$. 120

4.6 Test of significance of departure of Q =f(t) from Q = $aR + bR^2$ regression at -2.5 MPa KCl. 122

4.7 The respiration rates on a dry weight basis of P.cinnamomi, P.chrysogenum and C.fastidium grown on different potentials and osmotica comparing colonies of equal radius and of equal age. 127

4.8 The effect of the conditions of growth on isocitrate dehydrogenase extracted from P.cinnamomi assayed at different potentials produced with sucrose or KCl osmotica. 139

4.9 The effect of different solutes and osmotic potentials on NADP isocitrate dehydrogenase activity from Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium. 144

5.1 The effect of potential on wall thickness in Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium. 156

5.2 The effect of potential on cell size (diameter) in Phytophthora cinnamomi, Pencicillium chrysogenum and Chrysosporium fastidium. 157

5.3 Effect of osmotic potential on the morphology of Phytophthora cinnamomi tips. 175

5.4 Effect of osmotic potential on the morphology of *Penicillium chrysogenum* tips. 176

5.5 Effect of osmotic potential on the morphology of *Chrysosporium fastidium* tips. 177

5.6 A comparison of the colony dry weight to radius ratios of *Penicillium chrysogenum* and *Phytophthora cinnamomi* grown on media of osmotic potentials produced with KCl and with sugar. 178

5.7 Effect of osmotic potential on hyphal diameter in *Phytophthora cinnamomi* and *Chrysosporium fastidium*. 179

5.8 Time taken for regrowth to occur following osmotic shock to *Phytophthora cinnamomi*, *Penicillium chrysogenum* and *Chrysosporium fastidium*. 185

LIST OF PLATES

4.1 Electrolytic respirometer and pressure compensator. 107

5.1 Possible median sections through the apex of Phytophthora cinnamomi grown on 0 MPa and -10 MPa sucrose. 159

5.2 Possible median sections through the apex of Phytophthora cinnamomi grown on -2 MPa and -3 MPa sucrose. 160

5.3 Possible median section through the apex of Penicillium chrysogenum grown on -2.5 MPa KCl. 161

5.4 Plasmalemmasomes and lomasomes. 163

5.5 Mesosomes? 164

5.6 Glycogen granules around the margins of Penicillium chrysogenum cells (0 CYA) but absent leaving 'holes' after staining with uranyl acetate (-2.5 MPa glucose). 166

5.7 Glycogen. 167

5.8 Detail of β-glycogen rosettes showing 25 nm α particles in a section of Penicillium chrysogenum grown on 0 CYA. 168

5.9 Effect of hyperosmotic shock and hypoosmotic shock on Phytophthora cinnamomi. 184

5.10 Effect of different mountants on Chrysosporium fastidium hyphae grown on -1.25 MPa glucose. 189

1. GENERAL INTRODUCTION

Water relations of higher plants and microorganisms have gained considerable attention from both the practical and the academic standpoint. Filamentous fungi are of particular interest in this respect because they are able to survive and grow at water potentials lower than any other group. The literature on the subject has been recently and thoroughly reviewed e.g. Brown, 1976; 1978; 1979; Gutnecht et al., 1978; Zimmermann, 1978; Zimmermann & Steudle, 1978; Griffin & Luard, 1979; Griffin, 1980, to name but a few and does not need repeating here. However this literature spans fields which are very different in background and approach. I have therefore attempted to outline the theory behind this thesis and to relate it to the terminology of other disciplines. Some consideration is then given to the relationship between water and fungal ecology as a rationale for undertaking this work.

THE TERMINOLOGY OF WATER RELATIONS

Osmoregulation which is sometimes used as a more specific term for water relations is derived from the Greek ωσμος which is given in the Oxford dictionary as meaning 'push, thrust or impulse'. As this defininition implies, a force and hence energy is at the root of the matter, and a statement of principles must begin with thermodynamics. Even this treatment anticipates another review (Griffin, 1981).

The First Law of thermodynamics is a general statement about energy; the internal energy change (U) is the sum of the heat added to the system (Q) and the work done on the system (W):

$$\Delta U = Q + W \qquad (1)$$

where both Q and W are positive. Likewise, the Second Law is a general

statement about entropy, namely that in a reversible process, entropy (S) will remain constant, but in an irreversible process it will increase:

$$\Delta S = Q(rev) / T \qquad (2)$$

where Q(rev) is the heat added to the system and T the temperature. This can be rearranged to give an expression for pressure-volume work:

$$dU = TdS - PdV \qquad (3)$$

where T is the temperature, P the pressure and V the volume. A third function of state is the Gibb's free energy of the system (G) which is a measure of the spontaneity of the system alone:

$$G = H - TS = U + PV - TS \qquad (4)$$

where H is the enthalpy. Equations 3 and 4 can be used to derive an expression for the Gibb's free energy in a closed system:

$$dG = VdP - SdT \qquad (5)$$

In an open system, the number of moles (n) of substances in the system also becomes variable and an additional term must be considered. The total differential of the internal energy as a function of S, V and n_i becomes:

$$dU = (\partial U/\partial S)_{V,n_i} dS + (\partial U/\partial V)_{S,n_i} dV + (\partial U/\partial n_i)_{V,S,n_j} dn_i \qquad (6)$$

From equation 3, $(\partial U/\partial S)_{V,n_i}$ is equal to T, $(\partial U/\partial V)_{S,n_i}$ to -P and $(\partial U/\partial n_i)_{V,S,n_j}$ is defined as the chemical potential (μ_i) of the substance:

$$dU = TdS - PdV + \mu_i dn_i \qquad (7)$$

and

$$dG = VdP - SdT + \mu_i dn_i \qquad (8)$$

The chemical potential is therefore the equivalent of the partial molal Gibbs free energy of i ($\overline{G}$):

$$(\partial G/\partial n_i)_{T,P,n_j} = \mu_i = \overline{G}_i \qquad (9)$$

Having arrived at an expression for the chemical potential of a substance it now remains to relate it to water potential, the basic unit used here. If the ith component so far considered is taken to be water, the chemical potential of pure free water is the standard reference (μ_w°) and ($\mu_w - \mu_w^\circ$) is the chemical potential of the aqueous solution under consideration at the same temperature. This is a measure of the capacity of that system to do work and it therefore has units of energy (Joules/mole). The more convenient unit of energy per unit volume

(Joules/mole/m^3) is obtained by dividing by the partial molal volume of water ($\bar{V}_w$), and this expression is defined as the water potential (Ψ) of the system:

$$\Psi = (\mu_w - \mu_w^o)/\bar{V}_w \qquad (10)$$

Units of energy per unit volume are dimensionally equivalent to those of pressure and it is conventional to use this measure. The SI unit of pressure is the pascal (Pa) and a convenient derivative is the megapascal (MPa) which has been used throughout this work:

$$1 \text{ MPa} = 10^6 \text{ Pa}$$

A unit commonly used in earlier work was the bar where:

$$1 \text{ bar} = 10^5 \text{ Pa} = 0.1 \text{ MPa}$$

The water potential of a system can be considered as the sum of a number of constituent potentials which all act to alter the total potential from that of pure free water. These are the effects of temperature, gravity, the presence of interfaces between phases either solid-liquid or liquid-gas and repulsion and absorption (matrix) effects, the presence of dissolved solutes, and the presence of external pressure. In a system involving a microorganism and its environment, only the last three are usually considered and for practical purposes it is accepted that their effect on the total water potential is additive although this is certainly an oversimplification since there will be interactions between the terms:

$$\Psi = \psi_\tau + \psi_\pi + \psi_p \qquad (11)$$

where ψ_τ is the matric potential, ψ_π the osmotic potential and ψ_p the pressure potential. Different environments will be controlled by different types of potential, thus in soil the matric potential will be the dominant component, but in an aquatic medium it is of course insignificant and the water potential depends on the dissolved solutes or osmotic potential. The microorganism will have different constituent potentials but its total potential is assumed to be close to equilibrium with the environment.

There are other ways of quantifying the water status of a system, and their relationship to water potential may best be appreciated by considering the chemical potential in terms of the gas laws. The liquid phase of an aqueous system is in thermodynamic equilibrium with its gas

phase and the ideal gas law states that:

$$PV = nRT \tag{12}$$

where R is the universal gas constant. The partial molal volume of the ith component is therfore:

$$\overline{V}_i = RT/P_i \tag{13}$$

and P_i its partial pressure. $\overline{V}_i$ is also defined as $(\partial \mu_i/\partial P)_{T,n_i}$ so that in the case where i is water, by integrating we obtain an expression for the chemical potential in terms of its vapour pressure:

$$\mu_w - \mu_w^\circ = RT \ln p_w/p_w^\circ = RT \ln a_w \tag{14}$$

p_w/p_w° is the ratio of the partial gas pressure of the solution to that of the pure phase, the relative humidity if p_w° is taken as 100% or the water activity if p_w° is set at 1.0. Water activity is the basic unit of measurement in much microbiological work and in the stored food products industry, and it can be seen that it is related to water potential by its natural logarithm:

$$\Psi = (RT \ln a_w)/\overline{V}_w \tag{15}$$

Similarily Raoult's law may be applied which states that the partial pressure is equal to the mole fraction in an ideal solution:

$$p_w = N_w p_w^\circ \tag{16}$$

where N_w is the mole fraction of i. Thus again in the case of an ideal aqueous solution:

$$\psi_\pi = (RT \ln N_w)/\overline{V}_w \tag{17}$$

It is this relationship which leads to the common approximation that $-\ln N_w = N_s$ where $N_s = 1 - N_w$ and since $N_s/\overline{V}_w = c_s$ where c_s is the concentration of that solute:

$$\psi_\pi = -RTc_s \tag{18}$$

These relationships hold only for ideal and therefore dilute solutions. For the non-ideal solutions with which most of this study deals, an osmotic coefficient (ϕ) or an activity coefficient (γ) must be introduced, each of which is dependent on the temperature and the molality of the solute:

$$\mu_w - \mu_w^\circ = RT \ln \gamma N_w = RT\phi \ln N_w = RT \ln a_w \tag{19}$$

For an electrolyte an additional term must be added for the number of dissociating ions (v):

$$\psi_\pi = -RTmv\phi \tag{20}$$

where m is the molality. It is this relationship which has been used to

convert the concentrations given by other workers to water potential using published osmotic coefficients. The approximate water activity can also be calculated:

$$a_w = e^{-vm\phi / 55.51} \quad (21)$$

These concentrations are generally in terms of molarity (1000 cc solution) and not molality (1000 g solvent) and the difference between the two becomes important when the solution is concentrated. The same conversion can be used when concentrations have been given as percentages. The correct use of percentage as a concentration is grams solute/100 grams solution but other specifications such as w/v or v/v are sometimes given. Other measures of concentration in use are the osmolality and the freezing point depression. The former is defined as:

$$1 \text{ osmole/kg water} = -2.48 \text{ MPa} \quad (22)$$

and the latter:

$$\psi_\pi = -1.33\Delta T \text{ (MPa at } 25°) \quad (23)$$

where ΔT is the freezing point depression.

WATER RELATIONS AND FUNGAL ECOLOGY

The importance of morphology in the ecology and particularly in the water relations of microorganisms has been well described by Griffin (1972; 1978). His thesis is simply that single celled organisms, whether bacteria or fungal zoospores must rely on water filled pathways for movement and hence activity, and so are restricted to soils of a high water content. Filamentous fungi, by virtue of their growth form, are able to bridge air filled gaps and so tolerate far lower water contents and hence potentials. In an aquatic environment of course such distinctions do not apply. At the other extreme from the aquatic fungi are certain species which appear to be obligately xerophytic, and are able to grow at water potentials as low as -65 MPa. The majority of fungi however fall between these two extremes but are still widely tolerant of water stress so that the fungi of the physiologically dry regions of the world, whether desert or salt marsh, appear to be the

same species as those found in temperate soils of far higher average water content. The case of marine fungi is more complex and is probably related to reproductive requirements. The purpose of this study was to determine whether or not xerophily in fungi is a physiological aberration in a few species, or merely the extension of some general capability existing in most fungi to tolerate and adapt to various degrees of water stress, and if so to investigate how they achieve it.

It should of course be remembered that growth in pure culture is unlikely to be equivalent to that occurring in the natural environment. The latter probably occurs over a more limited range of water potential. Nevertheless pure culture conditions are a necessary preliminary in a physiological study such as this. The ability of fungi to use dissolved or gaseous oxygen makes it possible to culture them either submerged in liquid or on the still surface of a liquid or a solid medium. In surface growth, the phenomenon of apical growth and branching of hyphae produces the characteristic radial colonial growth form. In submerged liquid culture the hyphae intertwine so that a pellet form is produced. Small pellets are considered to be more homogeneous than the surface colony, also biomass production is greater and fungi of economic importance have been grown on a commercial scale by this method so that for these reasons liquid culture has generally been the method of choice for physiological studies. It has not been used here for two main reasons:

1. The difficulty of separating an organism grown in liquid culture from the growth medium, and the subsequent interference of contaminating liquid in analysis of the material.

2. The fungi used in this study are not found naturally in a liquid environment, and it has been shown that the degree of tolerance to low osmotic potential may depend upon the method of culture (Sterne et al., 1978).

Those who work on unicells such as the yeasts, microalgae or bacteria are obliged to use a liquid culture system, but the effects of

washing and subsequent error in analysis of the contents of such organisms is not always recognised. There are a number of reports where mycelial solutes have been analysed after extensive washing with distilled water and no account of the washings made (e.g. Ballio et al., 1964; Wright & Le Tourneau, 1965; Lahoz et al., 1966). Adler and Gustaffson (1980) have shown that polyols were lost from the yeast Debaryomyces hanseniit when washed, although Slayman and Tatum (1964) claimed that intracellular Na^+, K^+ and Cl^- of Neurospora crassa were not lost after washing. A number of workers have reported difficulty in analysis of ions in microorganisms grown in salt solutions (e.g. Borowitzka & Brown, 1974; Liu & Hellebust, 1976a; Aiking et al., 1977). Gimmler and Schirling (1978) had to go to great lengths to clean their organism by centrifugation through an oil of the appropriate viscosity in order to obtain accurate values for sodium content after growth in saline media. Therefore instead of liquid culture, the fungi used in this study have been grown entirely on solid media (agar plates) overlain with cellophane, which allows the colony to be separated from its growth medium with ease and the minimum of contamination. The water potential of agar media can conveniently be altered by controlling the osmotic component. Alteration of the matric potential of agar media is not a practical proposition.

The first task in this study was to measure osmotic and hence turgor potentials in a number of fungi, including the most xerophytic, growing over a wide range of potentials to determine whether or not turgor is regulated. Having established that it was, an analytical study was carried out to identify and quantify the various constituents of the cytoplasm responsible for the osmoregulation in three diverse species. Then some investigations were carried out on the energetics of osmoregulation, examining respiratory and enzymatic activity. Lastly the close connection between water potential and morphology was investigated in some detail.

† All species names mentioned in the text are listed with their authors in Appendix 1.

2. THE EFFECT OF WATER POTENTIAL ON FUNGAL GROWTH AND TURGOR

INTRODUCTION

Lowered external water potential usually reduces fungal growth which might be a result of reduced turgor. Accurate measurements of osmotic and turgor potentials of fungal hyphae are few, doubtless because of the limitations of the methods available. The micro-techniques called for by Robertson and Rizvi (1968) are still not to hand. An oil-filled capillary can be inserted into a cell and connected to a pressure transducer to measure turgor potential directly, but only if the cell is over 100 μm in diameter, so that most of such measurements have been on cells of the giant algae (Characeae) (Zimmermann et al., 1969). This technique has lately been improved so that cells of 20 μm can be used (Hüsken et al., 1978). This limit would include some higher plant cells but not the majority of fungal hyphae with the possible exception of the Saprolegniaceae. The coenocytic nature of the hyphae of this family is likely to make interpretation of pressure responses difficult.

Indirect means of determining fungal turgor potential have therefore still to be used. Assuming the fungus to be in water potential equilibrium with its growth medium, hyphal osmotic potential can be measured by various means and turgor potential found by subtraction using the relationships:

$$\psi_\pi^m = \Psi^h = \psi_\pi^h + \psi_p^h$$

where Ψ is the total water potential, ψ_π osmotic potential, and ψ_p turgor potential, all with units of pressure; m and h refer to medium and hyphae. Matric potential has been assumed to be negligible in fungal cells as in plant cells, although this is arguable and is discussed later. The theoretical limitations of these parameters have been considered by various authors (e.g. Brown, 1972; Dainty, 1976),

but as practical and measurable entities they still stand.

Early determinations of osmotic potentials whether by plasmolysis (Thatcher, 1939), acetic acid extrusion (Park & Robinson, 1966) or Robertson and Rizvi's (1968) tip morphology analysis, must be considered of doubtful value. More recently, psychrometry has been used for analysis of the components of water potential; there are now commercial instruments available which are suitable for small amounts of biological material.

Adebayo et al. (1971) used psychrometry to determine osmotic potentials in Mucor hiemalis Wehmer and Aspergillus wentii Wehmer grown at different water potentials. Colonies were grown on cheese cloth overlying an agar medium; for analysis the whole colony was scraped from the cheese cloth, frozen on dry ice, and then transferred to a psychrometer chamber. Höch and Mitchell (1973), working with the water mould Aphanomyces euteiches Drechs., used a liquid culture system. The mycelium was either disrupted in a homogeniser and a filter paper disc saturated with the resulting homogenate or the mycelial mat was dried on a Büchner funnel, and frozen directly in the psychrometer chamber. The results of these workers differ. The former found no correlation between fungal water potential and turgor potential; that is, approximately constant turgor potentials of 0.73 and 1.49 MPa respectively were maintained whatever the potential of the medium, although the growth limit of A.wentii was not tested. On the other hand, in A.euteiches turgor potential was negatively correlated with water potential, so that it was virtually zero at the limit of growth at -1.4 MPa.

Osmotic potentials can also perhaps be inferred from work on wall-less protoplasts of various species if it is assumed that they have been grown on dilute media with osmotic potentials of >-0.2 MPa. Eddy and Williamson (1957) found that protoplasts of the yeasts Saccharomyces carlsbergensis Hansen and S.cerevisiae Hansen were most stable in buffer solutions containing 0.55 molar rhamnose so that a potential of -1.6 MPa might be attributed to these two species. Similarly Neurospora crassa Shear & Dodge protoplasts were most stable in 0.59 molar sucrose (-1.8 MPa) (Bachmann & Bonner, 1959); Phytophthora cinnamomi Rands and

P.parasitica Dast. at -1.1 to -1.8 MPa (Bartnicki-Garcia & Lippman, 1966); and Penicillium chrysogenum Thom at -2.6 MPa (Anné et al., 1974).

It would appear then that fungi maintain an internal osmotic potential well below that of the growth medium. The present study using improved psychrometric techniques attempts to determine, in several species representing a wide range of tolerances to water stress, whether or not inability to maintain positive turgor is the cause of fungal growth limitation by low water potentials.

MATERIALS AND METHODS

Fungi

Phytophthora cinnamomi Rands mating type A1 (isolate 232) and A2 (isolate 176) were obtained from the culture collection of the Forestry Department, Australian National University; Phellinus noxius (Corner) G.H. Cunn. (116A) from L. Bolland of the same department; Fusarium equiseti (Corda) Sacc. (isolate F2186) came from L.W. Burgess, Department of Plant Pathology and Agricultural Entomology, University of Sydney; and Xeromyces bisporus Fraser (FRR 1533), Chrysosporium fastidium Pitt (FRR 77), Eurotium amstelodami Mangin (FRR 475), Penicillium chrysogenum Thom (FRR = NRRL 1951), and Aspergillus restrictus G. Smith (FRR 40) were all kindly supplied by J.I. Pitt, CSIRO Division of Food Research, North Ryde.

The growth medium was Czapek Dox to which 5 g/l Difco yeast extract was added (CYA); K_2HPO_4 was replaced by KH_2PO_4 to give a more acid medium (pH 5.8) which generally gives better growth of moulds (Smith, 1960). This medium was chosen in preference to the basal medium of Sommers et al. (1970) which has often been used for work on water potential, both because all the above species grow well on it, and because it is a rich medium (water potential -0.56 MPa). Sommers et al. (1970) observed Phytophthora parasitica to respond better to water

stress when grown on richer media such as V-8 agar (-0.25 MPa) or potato dextrose agar (-0.34 MPa), than on basal salt medium of -0.12 MPa.

C.fastidium and X.bisporus were maintained on CYA to which 400 g/l glucose had been added; E.amstelodami, P.chrysogenum, and A.restrictus on CYA supplemented with 200 g/l sucrose. P.cinnamomi was kept on V-8 agar, P.noxius on 2% malt extract agar and F.equiseti on carnation leaf agar (Tio et al., 1977).

Growth system

Separation of the fungus from its growth medium is a critical factor when measuring potentials, but one which is frequently overlooked. In liquid culture, allowance has to be made for medium which is trapped in the interstitial and apparent free space in the fungal pellets. Washing, even briefly in ice cold water, caused bursting of hyphal tips and loss of turgor, followed by rapid internal readjustment to a higher potential. Washing with non-metabolised isoosmotic solutions can also result in ion exchange, and the residue would still contribute to the potential. Removal of excess liquid by suction or centrifugation is not complete.

In this experiment colonies were grown on cellophane which completely covered the solid medium. The colony could be easily lifted or scraped off the cellophane, and being generally hydrophobic, appeared free of contaminating medium. Any moisture trapped between the colony and the cellophane could be removed by gently blotting between filter paper (Maclean & Scott, 1970, and pers. comm.). Commercial cellophane was prepared by boiling in deionised water for 30 min, and twice rinsed in distilled water before sterilising.

Plastic Petri dishes (9 cm) containing 30 ml medium were overlain with cellophane, and inoculated centrally with a 4 mm diameter plug cut from the margin of a colony whose growth rate was linear, or in the case of Aspergillus and Penicillium, from a spore suspension in 2% water agar. Plates were stored in plastic bags and incubated at 25°C. Radial growth rates were recorded at suitable intervals. Three radii were measured per plate, and there were four replicates of each osmotic

potential. Samples were taken for psychrometry while the growth rate was still linear.

Control of water potential

Water potential of the growth media was adjusted osmotically by the addition of solutes. Each species was tested with CYA adjusted with salt (KCl) or with a sugar (sucrose or glucose). The supposedly non-metabolized osmoticum, polyethylene glycol (PEG) was not employed because it is not soluble in agar solutions; also Mexal et al. (1975) have shown that oxygen diffusion through PEG solutions, at least of the larger molecular weights, is considerably reduced. Osmotic coefficients for KCl and sucrose were taken from Robinson and Stokes (1955); both these solutes form saturated solutions at about -22 MPa. Lower water potentials can be obtained with glucose, the solubility of which can be further increased by using a 1:1 glucose : fructose solution which saturates at 22.5 molal or -65 MPa. The water activities of glucose and fructose solutions were calculated from the equation of Norrish (1966). Water activities were converted to osmotic potentials using the relationship:

$$\psi_\pi = -RT/\overline{V}_w \ln a_w$$

where ψ_π is the osmotic potential, R and T have their usual meanings, $\overline{V}_w$ is the partial molal volume of water and a_w the water activity. Values for $\overline{V}_w$ were calculated for glucose and fructose solutions from densities published in the Handbook of Physics and Chemistry. This equation strictly holds only for dilute solutions. However actual values can be obtained experimentally by calibration against LiCl solutions, for which osmotic coefficients are published and osmotic potentials can be determined directly. The calibration with LiCl is shown in Fig. 2.1 together with the voltages obtained with glucose solutions of the same theoretical potential. The discrepancy between observed and expected values for glucose increases with decreasing potential to a difference of some 10 MPa at -50 MPa. The reasons for this are probably several, and include the complex effect of viscosity of concentrated sugar solutions on the rate of equilibration within the psychrometer chamber

(Greenwood, pers. comm.), the departure from ideality as already mentioned, and the experimental determination of the constant used in Norrish's equation. Potentials calculated from experimentally determined vapour pressures of glucose solutions published by Taylor and Rowlinson (1955) agreed fairly closely with those calculated from Norrish's equation; there was a difference of 0.2 MPa for a 14 molal solution.

Media above -5 MPa were sterlised by autoclaving at 121° for 15 min. More concentrated media were steamed for 30 min to prevent caramelization of sugars. Some browning did occur, particularly when glucose was sterilised at 121°. Although there is some evidence that the products of caramelization, such as hydroxy methyl furfural and formic acid, prevent fermentation by yeast (Ingram et al., 1955), browning did not appear to affect potential or growth. The pH of the medium was not altered by more than half a unit by the addition of large amounts of KCl or glucose, and so was not adjusted. Media above -4 MPa were dispensed with a syringe but more concentrated solutions were too viscous and had to be poured by hand. Precautions were taken to prevent water loss during preparation.

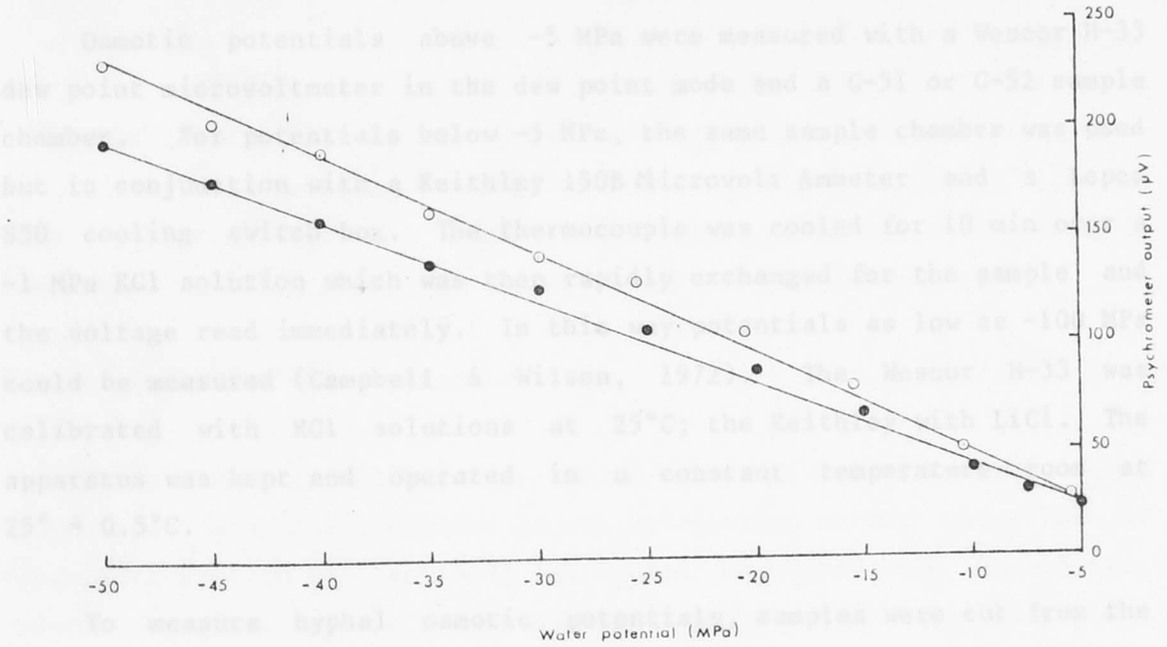


Fig. 2.1. Calibration of low water potential system with lithium chloride solutions (●) and experimental values for glucose solutions (○) of the same theoretical potential. Values are means of six measurements.

Psychrometry

Osmotic potentials above -5 MPa were measured with a Wescor H-33 dew point microvoltmeter in the dew point mode and a C-51 or C-52 sample chamber. For potentials below -5 MPa, the same sample chamber was used but in conjunction with a Keithley 150B Microvolt Ammeter and a Lepco S50 cooling switch box. The thermocouple was cooled for 10 min over a -1 MPa KCl solution which was then rapidly exchanged for the sample and the voltage read immediately. In this way potentials as low as -100 MPa could be measured (Campbell & Wilson, 1972). The Wescor H-33 was calibrated with KCl solutions at 25°C; the Keithley with LiCl. The apparatus was kept and operated in a constant temperature room at 25° ± 0.5°C.

To measure hyphal osmotic potentials, samples were cut from the margin of the colony, either with a 6 mm cork borer or scraped off the the cellophane with the back of a scalpel, depending on the consistency of the mat. The mycelium was blotted on filter paper and placed in the psychrometer sample holder; the whole wrapped in 'Parafilm' to prevent evaporation or condensation, and frozen for 10 min on dry ice. After thawing for 10 min, high potential samples were placed in the sample chamber and equilibrated for a further 10 min before reading. Low potential samples were thawed to 25° before being read. Twelve measurements were taken at each potential from the four replicate plates. Actual osmotic potentials of the media were taken as means of two or three measurements made at the time of preparation, before the agar was added.

RESULTS AND DISCUSSION

The results, in all the species tested, confirmed those of Adebayo et al. (1971) rather than those of Höch and Mitchell (1973). Lowered water potential, in all the various ranges, depressed and eventually suppressed radial growth, but turgor showed no such relationship.

Radial growth and water potential

Daily linear growth rates are shown in Fig. 2.2 A-H; the first measurement was not included in the calculation so that the effect of water potential on the length of lag phase has not been considered. Values represent surface growth over cellophane. Some comparisons were made of growth with and without cellophane (Table 2.1). Unexpectedly, cellophane appeared to stimulate growth, although in the case of P.cinnamomi this can be explained because the cellophane constrains the normal growth, which is predominantly through the agar, to the surface.

Table 2.1. Effect of cellophane on radial growth rate.

Species	External osmotic potential (-MPa)	Growth rate (mm/24 h) and standard deviations		Difference significance P = 0.001
		- Cellophane	+ Cellophane	
Phytophthora cinnamomi	0.07	7.7 ± 0.6	8.6 ± 0.4	+
Xeromyces bisporus	1.14	1.1 ± 0.1	1.4 ± 0.1	+
Xeromyces bisporus	4.16	0.9 ± 0.1	1.2 ± 0.1	+

The growth patterns reported here correspond with the distinctions made by Anand and Brown (1968) to define osmophilic and non-osmophilic yeasts. The xerophytic species such as X.bisporus and C.fastidium (Fig. 2.2H) showed only a gradual decline in growth as the water potential decreased. The non-tolerant species decreased sharply in growth rate after a much more clearly marked optimum. The basidiomycete P.noxius (Fig. 2.2C) was the only species which did not show the usual highly reproducible growth pattern. Growth was extremely erratic on both media, reflecting the tendency of this fungus to sector. However the overall trend was to slow and then stop growth in much the same range as P.cinnamomi. Maximum daily growth rates for all the species are shown in Table 2.2. The most xerophytic species were certainly also the slowest growing, but there was no clear demarcation between tolerant and non-tolerant species such as was noted between sugar tolerant and non-tolerant yeasts by Anand and Brown (1968).

Table 2.2. Maximum daily growth rates.

Species	External osmotic potential (-MPa)	Growth rate (mm/24 h)
Phellinus noxius	1.1	10.6
Fusarium equiseti	0.8	9.4
Phytophthora cinnamomi A2	1.1	8.8
Phytophthora cinnamomi A1	1.1	6.4
Eurotium amstelodami	11.2	5.2
Penicillium chrysogenum	2.9	4.1
Aspergillus restrictus	4.6	3.8
Chrysosporium fastidium	12.4	1.7
Xeromyces bisporus	26.5	1.7

As has often previously been observed there was some stimulation of

growth at potentials slightly below the highest tested. This effect has been attributed to an increase in energy supplied rather than a true osmotic effect (Adebayo & Harris, 1971) but this is unlikely where a rich medium has been used. Thus it might be supposed that hypotonic stress to mycelial growth can be imposed under certain field conditions as well as the more generally recognised hypertonic or drought stress. The obligately xerophytic species illustrate the extreme case. X.bisporus has an optimum at -20 MPa, C.fastidium at about -10 MPa and neither will grow above -5 MPa.

Anand and Brown (1968) considered that the nine strains of sugar-tolerant yeasts they tested were not obligately osmophilic. Only two of the nine did not grow on the basal medium, and this they thought to be due to an unsuitable medium rather than a true osmophilism. Quoting the work of Onishi (1960) on Torulopsis halonitratophila Onishi, they also argued that the apparent requirement for low water potential can be reversed by lowering the temperature, and they preferred to use the term xerotolerance. The effect of temperature on growth of xerophytic fungi has not been tested, although an increased tolerance of low water potential with increasing temperature has been reported for soil fungi causing cereal root-rots and cotton wilts (Manandhar & Bruehl, 1973; Cook & Christen, 1976).

Most of the species did not discriminate between sugar and salt in their growth response, indicating that the inhibition observed was an osmotic rather than a specific solute effect. E.amstelodami and F.equiseti were rather more sensitive to KCl than to sugar. The exceptions were the xerophytes X.bisporus and C.fastidium which did not grow on KCl adjusted media whatever the potential. NaCl, $CaCl_2$ and $MgCl_2$ were also tried with the same result. Pitt and Hocking (1977) obtained very limited growth with these two species on NaCl at pH 4.0 but none at pH 6.5. The two species have been isolated only from stored food products with a high sugar content, such as liquorice, dried prunes and chocolate sauce (Pitt, 1975). They have have not been found on natural sources probably because they are not selected by the usual isolation procedures. The other species are ubiquitous soil fungi; other workers have reported growth of such fungi as independent of the solute used (e.g. Sommers et al., 1970; Dubé et al., 1971; Manandhar &

Bruehl, 1973; Wilson & Griffin, 1979). Onishi (1963a) in his review of the osmophilic yeasts notes that the salt and sugar tolerances of different strains of the one species Saccharomyces rouxii Boutroux vary considerably according to their origin. For instance the soy yeasts were much less tolerant of NaCl than of sugar. These tolerances could to some extent be altered by training. Scott (1957) and Griffin and Luard (1979) both point out that definitions and distinctions between halophilism, osmophilism and xerophilism are arbitrary, and the water requirements of microorganisms probably show a continuous variation betwen species.

The obligate halophily of some bacteria has been explained on the basis of protein modification as well as internal ionic adjustment (Brown, 1976) and as far as we know there is no parallel in the fungi; the explanation for the various degrees of sugar and salt tolerances exhibited by other microorganisms depend on the ability to accumulate and store a compatible solute (Brown, 1978). Two possible explanations for the salt intolerance of the xerophytic fungi are that the ions fail to stimulate the necessary production of a compatible solute or that they alter membrane permeability and hence retention of such a solute. For instance Allaway and Jennings (1970b) have shown that sodium ions increase the loss of polyols from hyphae of Dendryphiella salina Nicot & Pugh while calcium ions decrease this loss.

Turgor and water potential

The mean values obtained for turgor potentials on both sugar and salt media calculated as the difference between medium water potential and hyphal osmotic potential are shown in Table 2.3, together with their standard deviations. The individual values are shown on Fig. 2.2A-H. There was no apparent decrease in turgor at the limit of growth in any of the species, nor were the values obtained when they were grown on KCl or sugar noticeably different.

It will be seen that the variation in the values is fairly large and is greater for the xerophytic species where the results were obtained using Campbell and Wilson's rapid exchange system. The largest standard deviation for the calibration of this technique was ± 5% at

-35 MPa; at this range the accuracy of reading was not better than ± 2.5 μV or 0.6 MPa. Undoubtedly, the short equilibration period contributed to the variability and possibly also accounts for the apparent trend of increasing turgor potentials with decreasing water potential. A similar trend was found by Adebayo et al. (1971) in Mucor hiemalis and by Boyer (1965) in cotton plants. If, as already mentioned, viscosity alters (presumably slows) equilibration rates, then the viscosity of the increasingly concentrated hyphae may well have a cumulative effect, giving abnormally 'dry' readings and so high turgor values. Wilson and Harris (1968) were unable to determine the potentials of seeds below -35 MPa by this method because equilibrium was not reached, although they considered that soils could be determined to -100 MPa.

Table 2.3. Average turgor potentials and standard deviations and the range of potentials tested.

Species	External osmotic potential range tested (-MPa)	Turgor potential (+MPa)
Phellinus noxius	0.37 - 3.63	1.01 ± 0.24
Phytophthora cinnamomi A1	0.37 - 3.63	1.02 ± 0.22
Phytophthora cinnamomi A2	0.49 - 3.67	0.86 ± 0.17
Fusarium equiseti	0.77 - 17.13	1.22 ± 0.60
Penicillium chrysogenum	0.41 - 26.93	2.14 ± 0.63
Eurotium amstelodami	5.43 - 41.49	3.13 ± 1.32
Aspergillus restrictus	5.70 - 36.22	2.45 ± 0.45
Chrysosporium fastidium	6.62 - 39.64	3.10 ± 1.29
Xeromyces bisporus	6.62 - 47.25	3.32 ± 0.62

F.equiseti forms profuse aerial hyphae which can be separated from those adpressed to the surface by gently scraping with a scalpel. There

was a difference of up to 1 MPa in the osmotic potentials of the two types (Fig. 2.2D). The greater turgidity of the aerial hyphae is to be expected if turgor performs the structural function of maintaining the hyphae erect. F.equiseti is the predominant type of Fusarium in arid zone pasture soils in New South Wales. It was more tolerant of water stress than other species of Fusarium investigated (e.g. Cook et al., 1972; Manandhar & Bruehl, 1973).

Hyphae from older parts of the P.chrysogenum colony had markedly higher osmotic potentials than those at the margin (Fig. 2.2E). Dead cells would lose their permeability characteristics and equilibrate with the medium with a resulting diluting effect. This effect was not observed when the same species was grown on 150 ml medium in 15 cm diameter glass Petri plates (unpublished results). The greater colony area to medium volume ratio in the smaller plates may have contributed to diffusion limitations on removal of staling factors or on the total available nutrient supply.

Turgor potentials in P.noxius did not show the same erratic behaviour as did the growth rates; thus an inability to osmoregulate does not appear to be the explanation for the variability of this species.

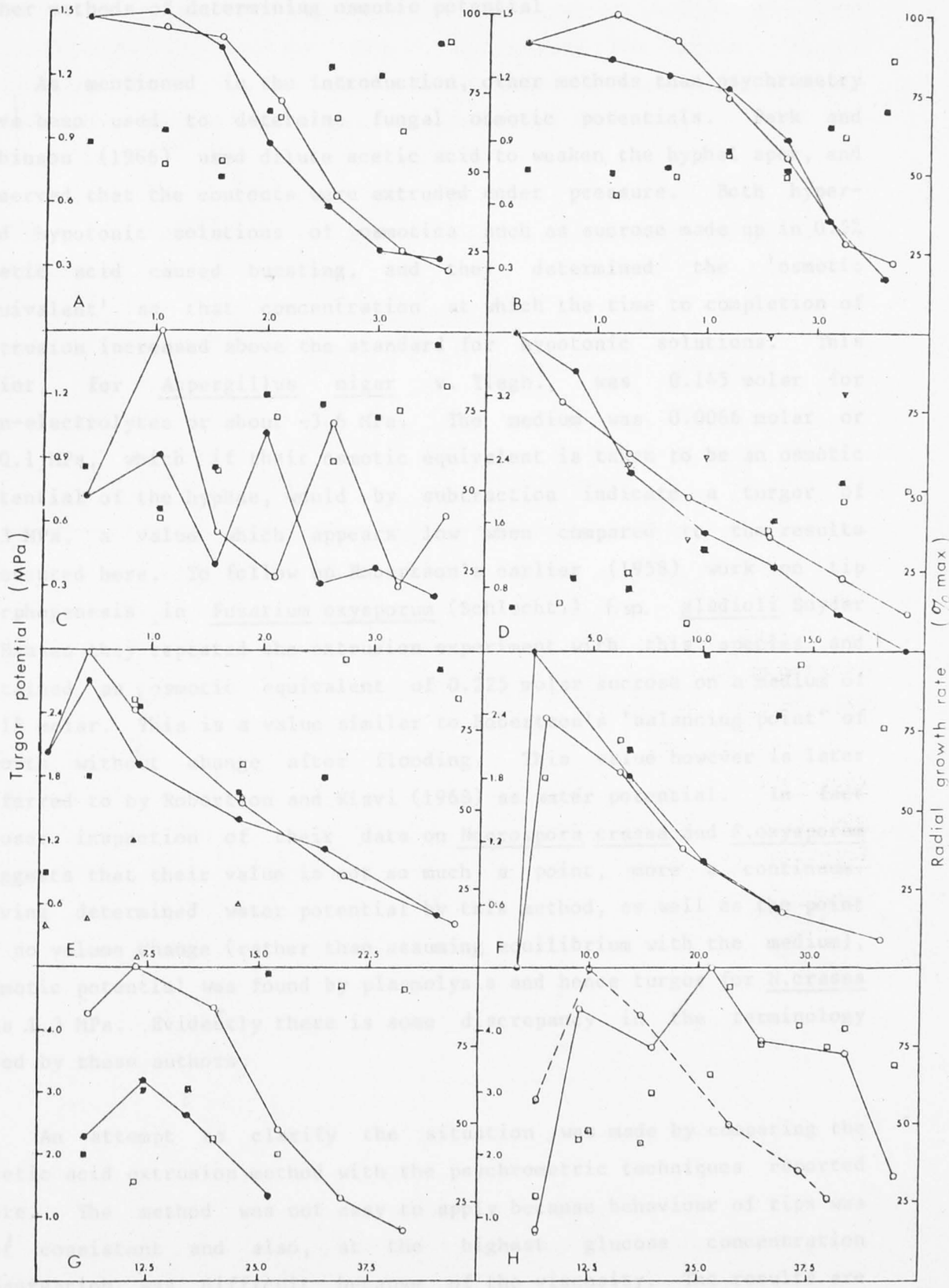
Fungal hyphae, at least the apices, differ from higher plants and multicellular algae in that they lack significant vacuoles. There is therefore no equivalent to the expressed sap sometimes used for osmotic determinations in the latter. Any expressed material would be cytoplasmic in nature; that is, solutes and organelles contained in a colloidal matrix of macromolecules. The distinction between matric (ψ_τ) and osmotic potential (ψ_π) in such a phase is arguable. In any case, psychrometric measurement of either disrupted tissue or expressed sap (because it contains some colloids), should be considered as a $\psi_{(\pi+\tau)}$ term. Expressing sap or cytoplasm will remove unknown proportions of water, solutes, and matrix from the tissue and so alter the potential. Both Boyer (1965) and Brown (1972) found a discrepancy of up to 0.3 MPa for $\psi_{(\pi+\tau)}$ of leaves measured on expressed sap and whole tissue. Thus the tissue method used here was considered more valid than expressing cytoplasm. The principal assumption then becomes that freezing does not

alter $\psi(\pi + \tau)$, which is probably not the case; there is likely to be an interaction of solutes with the cell wall fibres once the membranes are disrupted.

Several species of giant algae have been shown to maintain constant turgor when the potential of the external medium was altered although the absolute values varied considerably between species (Cram, 1976; Kirst & Bisson, 1979). Changes in ion transport in response to changes in internal osmotic or hydrostatic pressure appear to account for this turgor maintenance. Biochemical interconversions to produce compatible solutes are the predominant mechanism in other algae as well as the marine fungus Dendryphiella salina and the yeast Saccharomyces rouxii.

Undoubtedly the properties of the concentrated solutions used in these experiments are very different from the dilute and approximately ideal solutions normally encountered in plant water relations. Schobert (1977) considers that much intracellular water has properties which differ from those of free water and cannot act as a solvent for a compatible solute; a situation which would be enhanced by water removal under osmotic stress. She therefore postulates that compatible solutes such a polyols and proline are not functioning as osmoregulators as defined by Brown (1978) but as water-structure regulators.

Fig. 2.2. Effect of water potential on radial growth rates (circles) and turgor potentials (squares) of fungi grown on solid media adjusted with sugar (open symbols) and KCl (shaded symbols), unadjusted medium (▲); turgor potentials 2 cm behind margin of colony (△); aerial hyphae (▽). Values are means of twelve measurements. A. Phytophthora cinnamomi A1, B. P.cinnamomi A2, C. Phellinus noxius, D. Fusarium equiseti, E. Penicillium chrysogenum, F. Aspergillus restrictus, G. Eurotium amstelodami, H. Chrysosporium fastidium (- -), and Xeromyces bisporus (——).


Turgor potential (MPa)
Radial growth rate (% max)
A
B
C
D
E
F
G
H
Ψ_s (-MPa)

Other methods of determining osmotic potential

As mentioned in the introduction, other methods than psychrometry have been used to determine fungal osmotic potentials. Park and Robinson (1966) used dilute acetic acid to weaken the hyphal apex, and observed that the contents were extruded under pressure. Both hyper- and hypotonic solutions of osmotica such as sucrose made up in 0.5% acetic acid caused bursting, and they determined the 'osmotic equivalent' as that concentration at which the time to completion of extrusion increased above the standard for hypotonic solutions. This point, for Aspergillus niger v. Tiegh., was 0.145 molar for non-electrolytes or about -3 6 MPa. The medium was 0.0066 molar or >-0.1 MPa, which if their osmotic equivalent is taken to be an osmotic potential of the hyphae, would by subtraction indicate a turgor of 0.3 MPa, a value which appears low when compared to the results presented here. To follow up Robertson's earlier (1958) work on tip morphogenesis in Fusarium oxysporum (Schlecht.) f.sp. gladioli Snyder & Hansen they repeated the extrusion experiment with this species and obtained an osmotic equivalent of 0.225 molar sucrose on a medium of 0.15 molar. This is a value similar to Robertson's 'balancing point' of growth without change after flooding. This value however is later referred to by Robertson and Rizvi (1968) as water potential. In fact closer inspection of their data on Neurospora crassa and F.oxysporum suggests that their value is not so much a point, more a continuum. Having determined water potential by this method, as well as the point of no volume change (rather than assuming equilibrium with the medium), osmotic potential was found by plasmolysis and hence turgor for N.crassa was 1.3 MPa. Evidently there is some discrepancy in the terminology used by these authors.

An attempt to clarify the situation was made by comparing the acetic acid extrusion method with the psychrometric techniques reported here. The method was not easy to apply because behaviour of tips was not consistent and also, at the highest glucose concentration observation was difficult because of the viscosity. The results are shown in Table 2.4. In the case of P.noxius tips burst at potentials above those determined by psychrometry, and in one case at the same potential as the medium, and tip bursting probably does not represent

osmotic potential; in X.bisporus the result was not sufficiently clear to determine an absolute value. In summary the method, beyond suggesting that hyphae are under positive pressure which can be released by weakening the tip wall, cannot be considered rigorous.

Table 2.4. Comparison of hyphal osmotic potentials determined by psychrometry and tip bursting.

Species	External osmotic potential (-MPa)	Hyphal osmotic potential (-MPa)	
		Psychrometry	Tip bursting
Phellinus noxius	0.5	1.2	0.9
Phellinus noxius	3.6	4.9	3.6
Xeromyces bisporus	11.4	13.7	13.8-14.3

3. THE EFFECT OF WATER POTENTIAL ON HYPHAL SOLUTES

INTRODUCTION

The plant cell solutes which accumulate in response to drought or salt stress, whether they are considered to act as osmoregulators, compatible solutes, or both have been reviewed recently by Cram (1976) and Hellebust (1976). Subsequent to those reviews there have been a number of reports on osmoregulation. It has been shown that the quarternary ammonium compound glycine betaine and the imino acid proline were correlated with sap osmotic potentials in barley cultivars and in plants of salt marshes (Storey & Wyn Jones, 1977; Wyn Jones & Storey, 1978). The walled green alga Chlorella emersonii has been found to accumulate proline and sucrose (Greenway & Setter, 1979). In a freshwater isolate of another green alga, Stichococcus bacillaris, the levels of both proline and the polyol sorbitol (glucitol) varied with the salinity of the environment (Brown & Hellebust, 1978). The photosynthetic product digeneaside increased with external salinity in the marine red alga Griffithsia monilis (Bisson & Kirst, 1979) although the apparent concentration was too low to account for the observed internal osmotic potential; as with the other species mentioned ions also contributed. Organic compounds and the ions Na^+, K^+ and Cl^- were regulated to maintain constant turgor in several thalloid and giant celled marine algae (Kirst & Bisson, 1979). The halotolerant yeast Debaryomyces hansenii has been shown to accumulate glycerol by Adler and Gustaffson (1980), while Unemoto and Hayashi (1979) found glutamic acid and proline to respond to NaCl stress in the marine bacterium Vibrio alginolyticus.

Clearly much effort has been devoted to the understanding of osmotic or turgor regulation. It would seem that there is no single mechanism and that the variety of methods employed do not follow taxonomic criteria.

The filamentous fungi, as distinct from yeasts, have been little investigated in this respect. They have long been known to contain sugar alcohols or polyols, often in high concentrations, but only recently has an osmotic function been attributed to these compounds (Lewis & Smith, 1967). The glucose metabolism of the marine Hyphomycete Dendryphiella salina has been elaborated in some detail; in particular the polyols mannitol and arabitol have been shown to respond to high ion concentrations in the medium (Allaway & Jennings, 1970b), as have the hyphal cations (Allaway & Jennings, 1971). The conversion of soluble to insoluble carbohydrate, probably glycogen, which may be part of the osmoregulatory mechanism in this species has also been followed (Jennings & Austin, 1973; McDermott & Jennings, 1976).

Three of the eight species shown to maintain constant turgor in Chapter 2 were chosen for further study on the mode of this regulation. Phytophthora cinnamomi belongs to the lower fungi, which differ physiologically in a number of ways from all other fungi (Burnett, 1976). It has a mycelial growth limit of -4 MPa in culture although in the field this is likely to be too low a limit, and its wall-less motile zoospores are limited by very high water potentials (Duniway, 1979). It is an important plant pathogen, depending on moist soil for at least a a brief critical stage in its cycle. Penicillium chrysogenum is a Fungus Imperfectus although it may have Ascomycetous origins. It is a ubiquitous soil saprophyte, and like the other members of the Aspergillus and Penicillium group, has a wide tolerance to salt and osmotic stress. The limit for mycelial growth in culture is about -30 MPa. The third species, Chrysosporium fastidium, also a Fungus Imperfectus, is extremely xerophytic, probably for which reason it has only been recently described (Pitt, 1966). The only known habitats of this species are stored food products with a high sugar content. As well as being an obligate xerophyte growing in the range -5 to -45 MPa, it is unable to tolerate high salt concentrations.

Thus the three species represent the range of fungal tolerance to reduced water potential; they might be described as non-xerophytic, facultative and extremely xerophytic respectively. They are also taxonomically distinct and represent considerably different ecological behaviour. In the work described in this chapter, they have been grown

on solid media of a range of water potentials and the hyphae analysed for low molecular weight solutes in an attempt to identify the compounds which serve as osmoregulators or compatible solutes.

The first experiment repeats the steady state conditions used in Chapter 2. Hyperosmotic and hypoosmotic shock experiments were also carried out because accumulation may occur as an indirect result of stress under steady state conditions and it is necessary to demonstrate appearance or loss of solutes under shock conditions in order to implicate them in osmoregulation. Such experiments are easily accomplished with the experimental technique used for this work. Following the observation reported in Chapter 2 that turgor was lower in older parts of the colony, the contents of hyphae at different distances from the margin of large P.chrysogenum colonies were examined. Finally the discovery that P.cinnamomi employs a different method of regulation from the other two species prompted the examination of three other lower fungi.

MATERIALS AND METHODS

Fungi and media

Unless mentioned otherwise, Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium were maintained in culture and grown for experiments as described in Chapter 2. The media were also prepared in the same way. Actual total water potentials of all the media were not measured, so that for convenience, media are referred to throughout by the calculated osmotic potential produced by the added solute and represented by the symbol ψ_s. Thus a medium referred to as 0 MPa glucose will be the basal CYA with a true potential of -0.56 MPa, and belongs to a series of media to which glucose has been added to lower the osmotic potential. Similarily, -10 MPa KCl will have an actual potential close to -10.6 MPa.

Growth and dry weight

Radial growth was measured as described in Chapter 2. To obtain the fresh weight of a colony, the inoculum plug was removed and the colony lifted off the cellophane, blotted, and weighed in a tared aluminium foil boat. Dry weight was measured after drying at 85°C for at least a week. Dry weights of two whole colonies were used to calculate the dry weight of samples. If less than an entire colony was needed for analysis, sectors of the appropriate weight were cut. Replicate samples for the analyses specified below were taken from different colonies.

Hyphal osmotic potential

Hyphal osmotic potentials were determined as described in Chapter 2. Results were usually the means of three samples.

Cations

Samples of 100 to 150 mg fresh weight were weighed into crucibles and dry-ashed overnight at 420°C. The ash was extracted in 7 ml 3N HCl for 30 min over a boiling water bath. The residue was filtered through Whatman No 42 filter paper and made up to a known volume with distilled water. The extracts were analysed with a Varian Techtron AA5 atomic absorption spectrophotometer; Na^+ and K^+ by flame e mission, Ca^{2+} and Mg^{2+} by atomic absorption. Results were the means of three samples. Fresh standards were prepared for each experiment, and the analyses for Na^+ and K^+ were carried out the day that the extracts were prepared.

Chloride

Samples of 150 to 250 mg fresh weight were placed in 15 ml centrifuge tubes and frozen in dry ice for 20 min. After thawing, 1 or 1.5 ml distilled water was added and the tubes shaken at room temperature for 1 h. Aliquots of 25 to 100 µl of the water extract were titrated using a Radiometer CMT 10 chloride titrator. Results were the means of at least three samples.

*

Water and ethanol have different boiling points so that the concentration of the mixed solvent in the extraction thimble of the Soxhlet apparatus would have been greater than 80% ethanol. While sugars and polyols are generally soluble in 95% ethanol and are likely to have been completely extracted by this procedure, there is a possibility that some of the amino acids which are less soluble in ethanol, such as glutamic acid, aspartic acid, glycine and alanine may not have been completely taken up.

Extraction for organic analysis

Approximately 400 mg fresh weight of colony was rapidly killed with 20 ml hot absolute ethanol and extracted in 100 ml 80% (v/v) ethanol in a Soxhlet apparatus for 24 h*. The extract was dried in a rotary evaporator at 40°C under reduced pressure. The residue was taken up in 4 ml 80% ethanol and divided equally for carbohydrate and amino acid analyses. The portions, in 25 ml pear shaped flasks, were again dried on a rotary evaporator and stored overnight with phosphorus pentoxide to ensure complete dryness.

All solvents were redistilled before use. No further clean up of the ethanol extracts was found to be necessary.

Amino acids

Dried samples from the 80% ethanol extracts were taken up in 1 ml sodium citrate buffer pH 2.2. Aliquots, usually 100 µl, were analysed on a model 119 CL Beckman amino acid autoanalyser. The peaks were integrated by a model 126 Data System. In some of the earlier experiments samples were analysed on Beckman models 120B or 120C belonging to a different unit. In this case the stock volume was 2 or 2.5 ml and some of the peak areas were estimated by hand as height times width at half height. Results were from single samples except in the case of the P.cinnamomi steady state series where they were in duplicate.

Carbohydrate

Ethanol soluble carbohydrates were analysed by gas-liquid chromatography using the method of Holligan and Drew (1971) with some modifications. The instrument used was a Perkin Elmer F11 flame ionisation gas chromatograph; the copper columns (1.8 m long, 1.5 mm internal diameter) contained 2% SE 52 stationary phase on acid washed Chromosorb W solid support. The carrier gas was high purity nitrogen supplied at 40 ml/min. Air and hydrogen were supplied at 300 ml/min and 30 ml/min respectively. There was no separate heater for the detectors so that they were always at the same temperature as the oven. The

detectors were cleaned at frequent intervals to remove ash which accumulated and reduced the detector response.

Trimethyl silyl (TMS) volatile derivatives of the soluble carbohydrates were prepared as follows: the sample was dissolved in about 2 ml anhydrous pyridine (stored over KOH and with molecular sieve), heated for a few minutes to dissolve solids if necessary, 0.5 ml hexamethyldisilizane and 0.25 ml trimethylchlorosiliane were added, the flask was shaken vigorously for 30 sec and allowed to stand at room temperature for 3 h. The slow elution of pyridine precluded its use as the solvent on the column because it completely masked the early peaks. The pyridine was therefore evaporated under reduced pressure at 30° and the derivatives redissolved in 0.5 ml n-hexane. This also had the advantage of excluding salts, which were insoluble in hexane, so that the high concentrations of K^+ and Cl^- anticipated in some of the samples did not interfere with the chromatogram. In the P.cinnamomi samples, substances insoluble in hexane did mask the chromatogram, and it was necessary to centrifuge the samples and inject the supernatant only.

Samples of up to 1 µl were injected using an SGE series 1B syringe with a 7 cm needle and fitted with a repeating adaptor. The temperature of the injection ports was 250°C. A Hitachi, and subsequently a Gurken, chart recorder was used. Operating conditions were as follows:

compound	temperature programme	amplifier range	recorder chart speed
glycerol	100° isothermal	50×10^2	1 cm/min
erythritol, arabitol, fructose, glucose, mannitol	100°- 250° 4°/min	10×10^2	0.5 cm/min
sucrose	150°- 250° 10°/min	20×10^2	0.5 cm/min

There was no compound suitable to use as an internal standard in the P.chrysogenum and C.fastidium extracts. Ribose was the only compound not present in the samples with a suitable retention time, but it was resolved into at least four peaks when hexane was the secondary solvent. The instrument was therefore calibrated with sucrose (10 µg/µl), a mixture of erythritol, arabitol, fructose, glucose and mannitol (5 µg/µl each), and glycerol (10 µg/µl) under the conditions mentioned. Standards were made up in 80% ethanol, heated to equilibrate sugar isomers, and treated in the same way as the samples; 0.2 to 1.0 µl was injected.

Amounts were calculated from peak areas which were determined as peak height times width at half height. A Bausch and Lomb micrometer magnifying lens (7x) was used to measure peak width. Identification of peaks was by coincidence with authentic standards. TMS derivatives do not separate the isomeric pentitols arabitol, ribitol or xylitol, or the hexitols mannitol, galactitol or glucitol. Acetate derivatives will separate the pentitols, but not the hexitols in the presence of glucose (Holligan & Drew, 1971). The presumed presence of mannitol and arabitol were therefore confirmed by paper chromatography using the method of Robyt (1975), except that the chromatogram was run for 24 h at 17°C.

As the purpose of the investigation was to identify low molecular weight compounds in the hyphae, the chromatogram was stopped after the elution of the monosaccharides except when sucrose was analysed in P.cinnamomi. Trehalose was present in all three species in small amounts. TMS derivatives of organic acids prepared in this way were not stable and could not be estimated. A compound which was probably malic acid co-chromatogrammed with erythritol in the P.chrysogenum KCl series.

THE EFFECT OF STEADY STATE OSMOTIC STRESS ON HYPHAL SOLUTES

Phytophthora cinnamomi was grown on CYA the osmotic potential of which had been lowered, in one series with KCl and in a second series with sucrose, to -2.5 MPa. *Penicillium chrysogenum* was tested on potentials down to -10 MPa produced with glucose or KCl. *Chrysosporium fastidium* was tested on glucose media only and the lowest potential was -20 MPa. Media of -17.5 and -20 MPa were made by mixing equal molalities of glucose and fructose as described in Chapter 2.

Colonies were grown at 25°C and the weight and colony radius were measured at suitable intervals; daily for *P.cinnamomi*, every 2 days for *P.chrysogenum*, and 4 days in the case of *C.fastidium*. When the colonies had reached a radius of at least 20 mm, samples were taken for analysis of carbohydrate, cations, chloride, amino acids, water content and osmotic potential as described above.

RESULTS

Hyphal water content and osmotic potential

The effect of growth at different external osmotic potentials on the hyphal osmotic potential and the fresh weight (fw) to dry weight (dw) ratios of the three species is shown in Fig. 3.1. Hyphal osmotic potentials agree with those discussed in Chapter 2, and positive turgor was maintained under all conditions.

The water content per unit dry weight decreased with the potential in both *P.cinnamomi* and *P.chrysogenum*; by a maximum of 40% in the *P.chrysogenum* glucose series and 24% in the *P.cinnamomi* KCl series. At an equivalent potential of -2 MPa the water content of *P.cinnamomi* was slightly less than that of *P.chrysogenum*. The rather erratic values of

the P.cinnamomi KCl series probably reflect the very low biomass production. Also, P.cinnamomi colonies were markedly less hydrophobic than the other two species, so that there may have been some contamination by interstitial water despite the blotting procedure.

The effect of water potential on water content was less clear in C.fastidium. Over a range of 15 MPa there appeared to be little, if any, change in water content. The water content was never lower than the lowest value observed in P.chrysogenum; on equivalent media of both -5 and -10 MPa glucose the water content of P.chrysogenum was lower than C.fastidium. The fact that P.chrysogenum samples always contained conidia, which were not present in the other two species, should also be taken into account. The water content of spores has often been assumed to be lower than vegetative mycelium, implying a lower potential in the former (Gould & Measures, 1977) although throughout this work the assumption has been made that the two are in equilibrium (Cochrane, 1958).

In the first two species, the decrease observed in hyphal osmotic potential must therefore be at least in part due to an increase in concentration of solutes in the presence of reduced solvent.

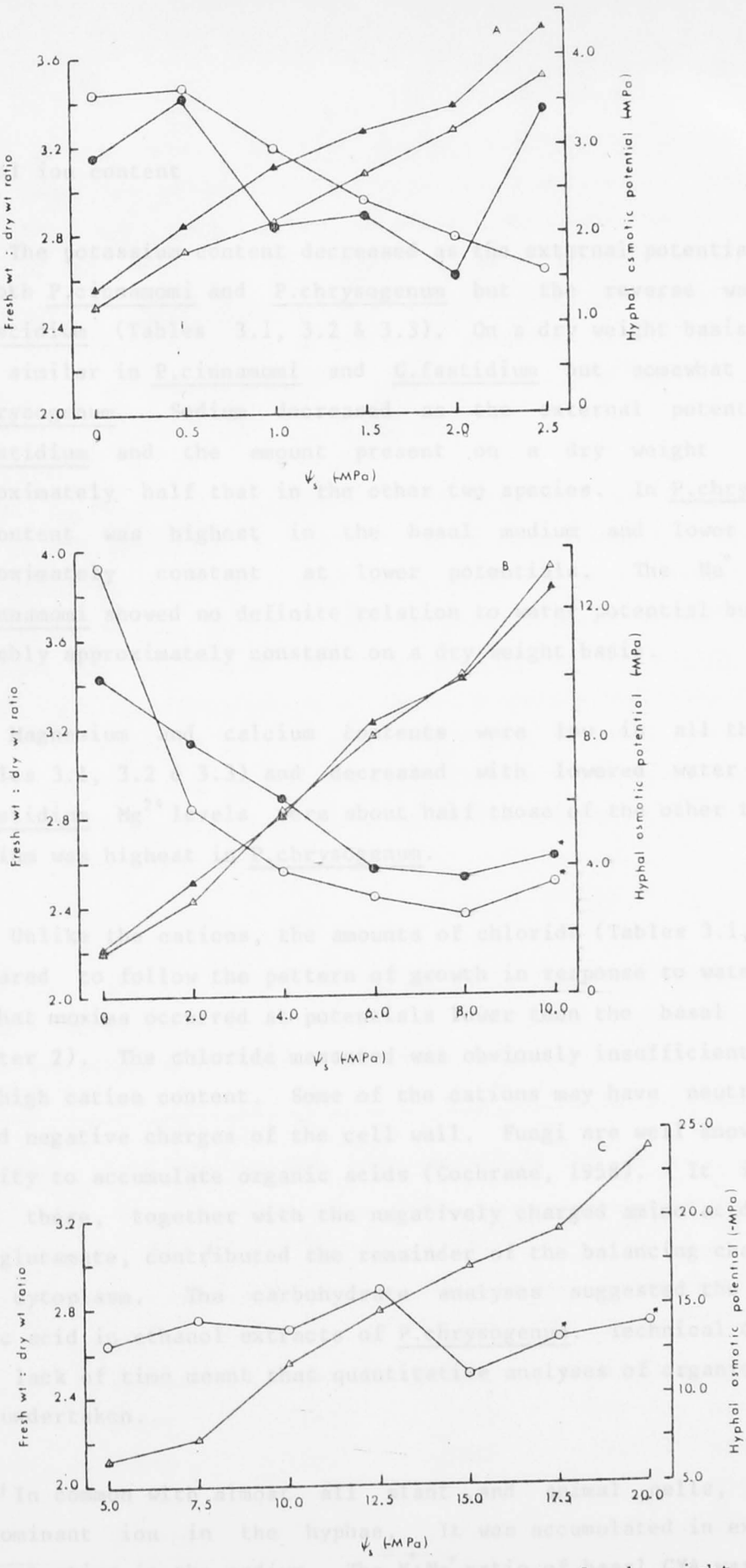


Fig. 3.1. Effect of water potential on the fresh weight to dry weight ratios (circles) and hyphal osmotic potentials (triangles) of colonies (20 mm radius) of Phytophthora cinnamomi (A), Penicillium chrysogenum (B) and Chrysosporium fastidium (C) grown on sugar osmotica (open symbols) and KCl (shaded symbols). * Colony <20 mm.

Hyphal ion content

The potassium content decreased as the external potential decreased in both P.cinnamomi and P.chrysogenum but the reverse was true in C.fastidium (Tables 3.1, 3.2 & 3.3). On a dry weight basis K^+ contents were similar in P.cinnamomi and C.fastidium but somewhat higher in P.chrysogenum. Sodium decreased as the external potential fell in C.fastidium and the amount present on a dry weight basis was approximately half that in the other two species. In P.chrysogenum the Na^+content was highest in the basal medium and lower (65%) and approximately constant at lower potentials. The Na^+ content of P.cinnamomi showed no definite relation to water potential but was also probably approximately constant on a dry weight basis.

Magnesium and calcium contents were low in all three species (Tables 3.1, 3.2 & 3.3) and decreased with lowered water potential. C.fastidium Mg^{2+} levels were about half those of the other two species. Calcium was highest in P.chrysogenum.

Unlike the cations, the amounts of chloride (Tables 3.1, 3.2 & 3.3) appeared to follow the pattern of growth in response to water potential in that maxima occurred at potentials lower than the basal media (see Chapter 2). The chloride measured was obviously insufficient to balance the high cation content. Some of the cations may have neutralised the fixed negative charges of the cell wall. Fungi are well known for their ability to accumulate organic acids (Cochrane, 1958). It is probable that these, together with the negatively charged amino acids aspartate and glutamate, contributed the remainder of the balancing charge within the cytoplasm. The carbohydrate analyses suggested the presence of malic acid in ethanol extracts of P.chrysogenum. Technical difficulties and lack of time meant that quantitative analyses of organic acids were not undertaken.

In common with almost all plant and animal cells, K^+ was the predominant ion in the hyphae. It was accumulated in excess of the concentration in the medium. The K^+:Na^+ ratio of basal CYA was close to unity. The K^+:Na^+ ratios obtained for the three species when grown with a sugar osmoticum are shown in Fig. 3.2 and have been related to the

radial growth rate. In P.chrysogenum and P.cinnamomi the ratios and growth rates follow similar patterns in response to lowered water potential, with optima below the highest potential tested. The actual values of the ratios in P.chrysogenum were almost twice those for P.cinnamomi. C.fastidium did not show this pattern; there was a linear increase in the K^+:Na^+ ratio as the water potential fell. This appeared to be due both to increased K^+ accumulation and Na^+ exclusion. All the values exceeded those for P.chrysogenum.

The K^+, Na^+ and Cl^- contents of P.chrysogenum and P.cinnamomi when grown on KCl are shown in Fig. 3.3. Both accumulated K^+ in proportion to the concentration in the medium and also Cl^- but to a lesser extent. The difference between K^+ and Cl^- was approximately constant in both species and about twice as large in P.chrysogenum as in P.cinnamomi, thus leaving a greater proportion of negative charges unaccounted for in the former. Na^+ remained at low levels which were slightly lower than when either species was grown on sugar (Tables 3.1 & 3.2). No consistent differences were noticed in Mg^{2+} or Ca^{2+} levels between treatments in either species (values not shown).

Table 3.1. The ion content of Phytophthora cinnamomi grown at different osmotic potentials produced by the addition of sucrose. Values are means and standard deviations of three samples.

Ψ_s (-MPa)	Ion content (μequivalents/g dw)				
	K^+	Na^+	Mg^{2+}	Ca^{2+}	Cl^-
0	368±19	105±3	23±2	3±1	73±16
0.5	304±29	82±3	19±1	3±1	83±6
1.0	303±5	111±20	19±1	1±1	82±12
1.5	186±0	75±1	12±2	1±1	58±5
2.0	220±20	107±10	14±1	1±0	69±5
2.5	240±26	112±2	15±2	1±0	81±4

The presentation of ion contents on a dry weight basis may be misleading since there will be a considerable increase in weight (i.e. density) of hyphae due to accumulation of osmotic solutes as the external potential is reduced. For example, a 4 molal solution of glycerol necessary to produce a hyphal osmotic potential of -10 MPa would contribute 368 g/kg cell water or more than 50% of the dry weight. Any error will presumably be most noticeable at the lowest potential tested, that is for C.fastidium. Since the internal osmotica were not entirely accounted for in P.chrysogenum and C.fastidium (see discussion at the end of the chapter), it may be more informative to recalculate the ion contents of these two species in terms of dry weight minus theoretical osmotic solutes. Potassium contents have therefore been calculated on a dry weight basis from which the weight due to the theoretical amount of glycerol needed as a hyphal osmoticum for each external potential has been subtracted. Column 7 in Tables 3.2 and 3.3 now suggests that the K^+ content increases to very high levels at low potential. However this treatment is presumably oversimplified, since the calculation suggests that C.fastidium colonies grown at potentials below -17.5 MPa contain insufficient water to produce the theoretical potential with glycerol and one arrives at the situation of more than 100% of the dry weight attributable to glycerol.

Some other non-osmotic parameter such as protein or nucleic acid might provide a more meaningful basis. Since a colony grows on solid media as a relatively flat disc, a unit area basis is an alternative, assuming that potential does not alter the depth or density of the colony. The K^+:Na^+ ratios for P.chrysogenum and C.fastidium calculated on this basis are shown in the final column of Tables 3.2 and 3.3. In fact similar relationships are observed to those shown in Fig. 3.2. A similar argument could be applied to the results of the osmotic shock experiments (Tables 3.8 and 3.9). However although recalculation of the K^+:Na^+ ratios on a unit area basis increases the values, it does not alter the trends observed.

Table 3.2. The ion content of Penicillium chrysogenum grown at different osmotic potentials produced by the addition of glucose. Values are means and standard deviations of three samples.

Ψ_s (-MPa)	Ion content (μequivalents/g dw)					K^+*	$K^+:Na^+$**
	K^+	Na^+	Mg^{2+}	Ca^{2+}	Cl^-		
0	510±92	101±32	29±5	16±6	41±6	510	8.4
2.0	413±18	60±3	21±3	68±4	114±8	478	11.2
4.0	444±9	68±4	19±1	7±2	90±8	577	10.3
6.0	400±17	72±4	18±1	8±2	80±5	589	9.3
8.0	392±20	64±5	18±1	6±3	59±3	658	10.0
10.0	389±17	65±16	18±2	4±2	53±2	878	7.5

Table 3.3. The ion content of Chrysosporium fastidium grown at different osmotic potentials produced by the addition of glucose. Values are means and standard deviations of three samples.

Ψ_s (-MPa)	Ion content (μequivalents/g dw)					K^+*	$K^+:Na^+$**
	K^+	Na^+	Mg^+	Ca^+	Cl^-		
5.0	278±10	35±1	12±0	2±1	46±2	399	13.5
7.5	296±25	38±1	12±0	1±1	51±1	577	13.3
10.0	302±51	32±2	10±1	1±1	58±6	818	15.7
12.5	306±2	28±0	10±1	1±0	55±6	2391	18.3
15.0	308±5	27±0	10±0	1±1	62±2	1855	19.6
17.5	330±9	25±3	6±0	1±1	65±2	-	21.7
20.0	345±9	23±0	6±0	0±0	88	-	24.3

* μequivalents/g dw where the weight of glycerol theoretically needed to balance external potential has been subtracted; no allowance made for non-osmotic water.

** Calculated on a unit area basis.

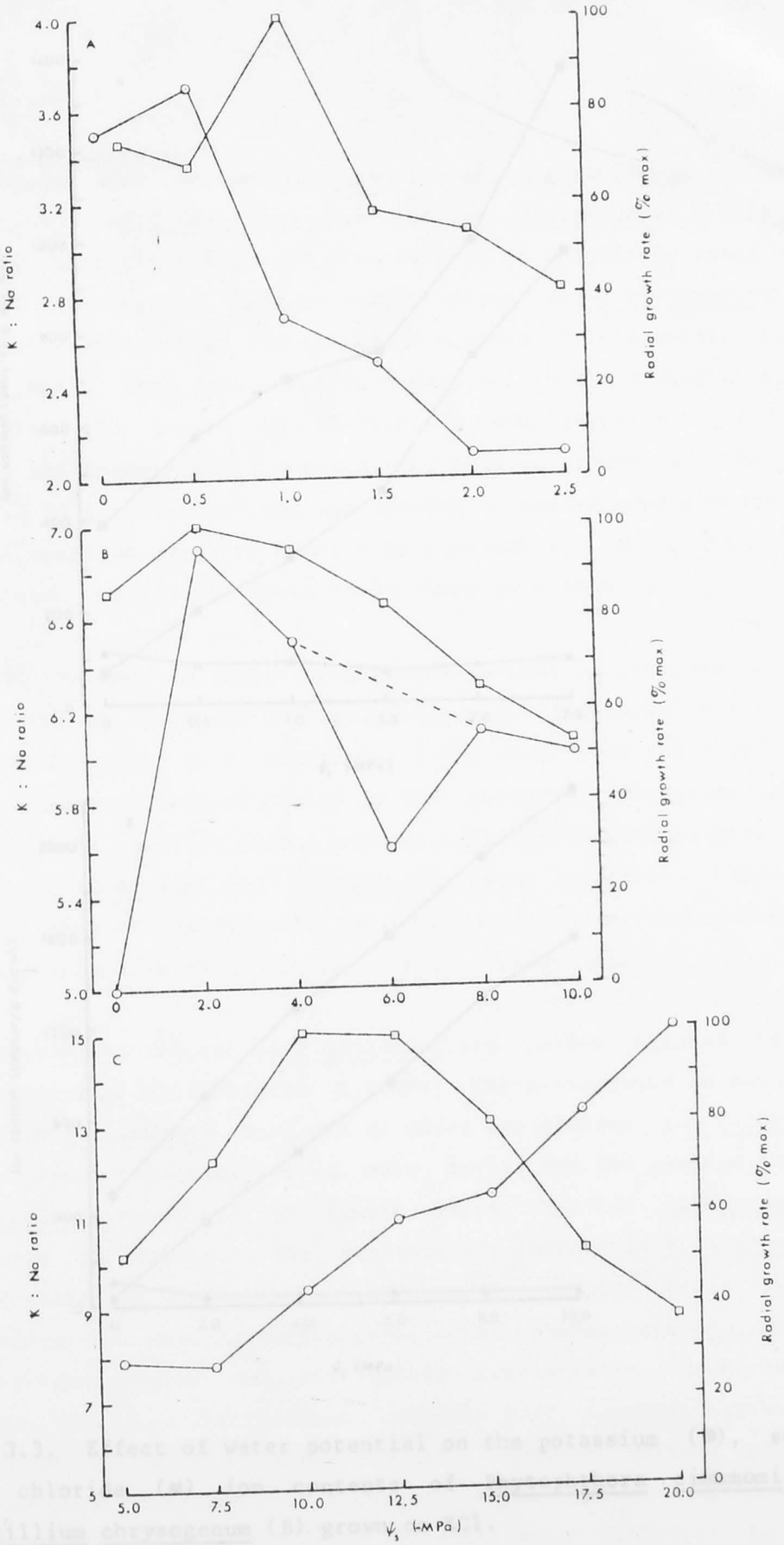


Fig. 3.2. Effect of water potential on the potassium to sodium ratios (○) and the radial growth rates (□) of Phytophthora cinnamomi (A), Penicillium chrysogenum (B) and Chrysosporium fastidium (C) grown on sugar osmotica.

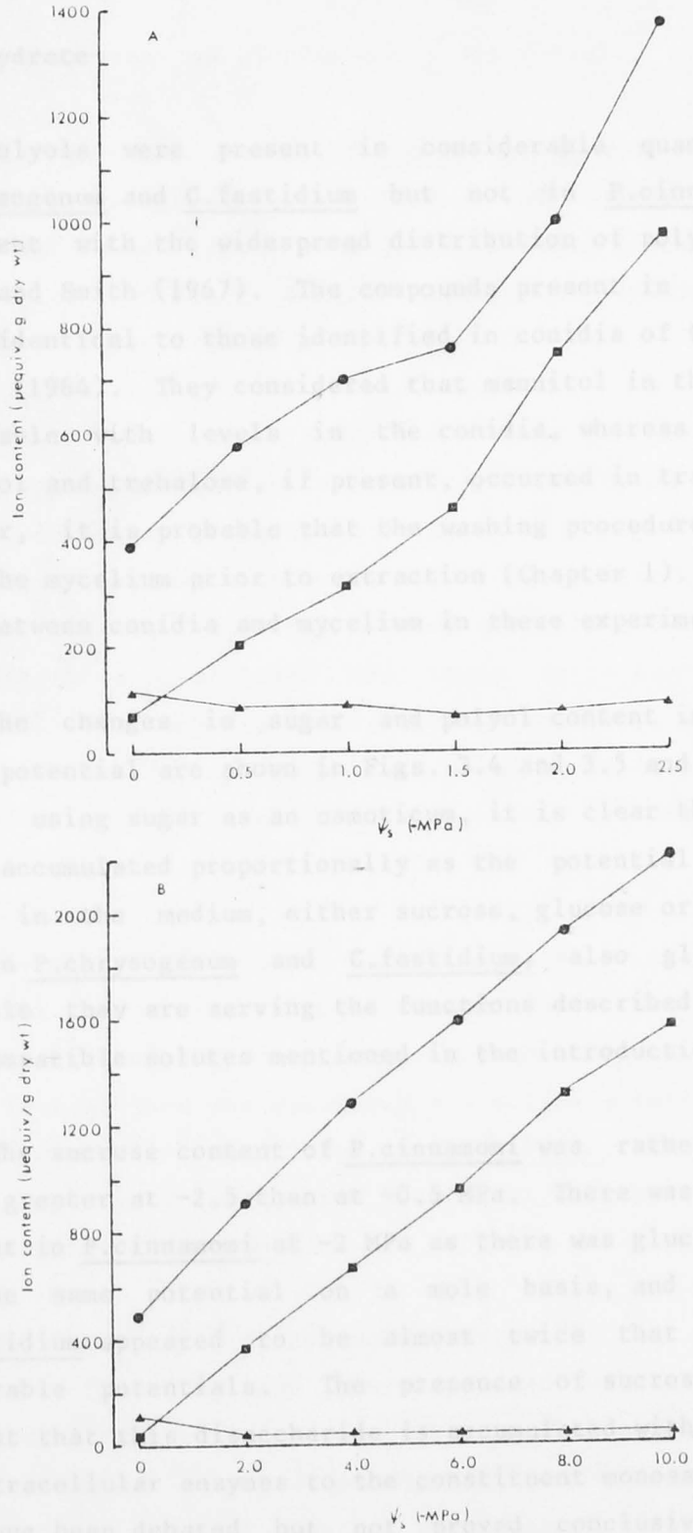


Fig. 3.3. Effect of water potential on the potassium (●), sodium (▲) and chloride (■) ion contents of Phytophthora cinnamomi (A) and Penicillium chrysogenum (B) grown on KCl.

Carbohydrate

Polyols were present in considerable quantity in the hyphae of P.chrysogenum and C.fastidium but not in P.cinnamomi. This is in agreement with the widespread distribution of polyols in fungi noted by Lewis and Smith (1967). The compounds present in P.chrysogenum appear to be identical to those identified in conidia of this species by Ballio et al. (1964). They considered that mannitol in the washed mycelium was comparable with levels in the conidia, whereas glycerol, erythritol, arabitol and trehalose, if present, occurred in traces in the former. However, it is probable that the washing procedure used removed polyols from the mycelium prior to extraction (Chapter 1). No distinction was made between conidia and mycelium in these experiments.

The changes in sugar and polyol content in response to external water potential are shown in Figs. 3.4 and 3.5 and in Table 3.4. In the series using sugar as an osmoticum, it is clear that the only compounds which accumulated proportionally as the potential decreased were the sugar in the medium, either sucrose, glucose or glucose plus fructose and, in P.chrysogenum and C.fastidium, also glycerol. Thus it is probable they are serving the functions described as the osmoregulators and compatible solutes mentioned in the introduction.

The sucrose content of P.cinnamomi was rather erratic being not much greater at -2.5 than at -0.5 MPa. There was twice as much sucrose present in P.cinnamomi at -2 MPa as there was glucose in P.chrysogenum at the same potential on a mole basis, and the glucose content of C.fastidium appeared to be almost twice that of P.chrysogenum at comparable potentials. The presence of sucrose in P.cinnamomi would suggest that this disaccharide is accumulated without prior hydrolysis by extracellular enzymes to the constituent monosaccharides. This point has long been debated but not proved conclusively (Cochrane, 1958; Jennings, 1976). The glucose contents of P.cinnamomi hyphae whether grown on KCl or sucrose were similar. However neither glucose or fructose were detected when P.chrysogenum was grown on media where KCl was the osmoticum. Low levels of fructose were difficult to measure accurately because at least three peaks were present in the TMS derivatives, but small amounts were present in both P.chrysogenum and

C.fastidium grown on glucose (data not shown).

The glycerol content of P.chrysogenum when colonies had been grown on media containing KCl was half that of hyphae taken from colonies which had been grown on media with added glucose. P.chrysogenum and C.fastidium contained similar amounts of glycerol when grown at equivalent potentials of glucose.

The other polyols present, that is mannitol, arabitol and erythritol do not appear to be acting directly in this way. All three showed different patterns in response to lowered osmotic potential. In the three experiments shown in Fig. 3.5, the mannitol level was highest at the highest potential tested and fell as the potential decreased. Although the initial level was lower in C.fastidium, if equivalent potentials are compared the values are similar. As this is the converse of the pattern for osmoregulatory solutes, it is possible that mannitol is acting as a reserve for glycerol synthesis or is implicated in glucose uptake.

The erythritol present in P.chrysogenum showed a maximum at a potential below the highest tested and then declined, although the pattern differed depending on the external solute. The levels were also twice as high when the osmoticum was KCl as when it was glucose. Traces only of erythritol were found in C.fastidium. It is therefore possible that there is a correlation between erythritol content and growth rate at least in P.chrysogenum.

The levels of arabitol were fairly low in the three treatments shown in Fig. 3.5, and there appeared to be a slight increase as the potential decreased. It is possible then that this compound is acting as a minor or 'second line compatible solute suggested by Brown (1978) for the sugar tolerant yeasts. The presence of equi-molal fructose and glucose which was necessary to produce potentials below -15 MPa stimulated the production of both mannitol and arabitol but not glycerol in C.fastidium, which may be further evidence that the last responds to the potential while the first two are metabolic by-products.

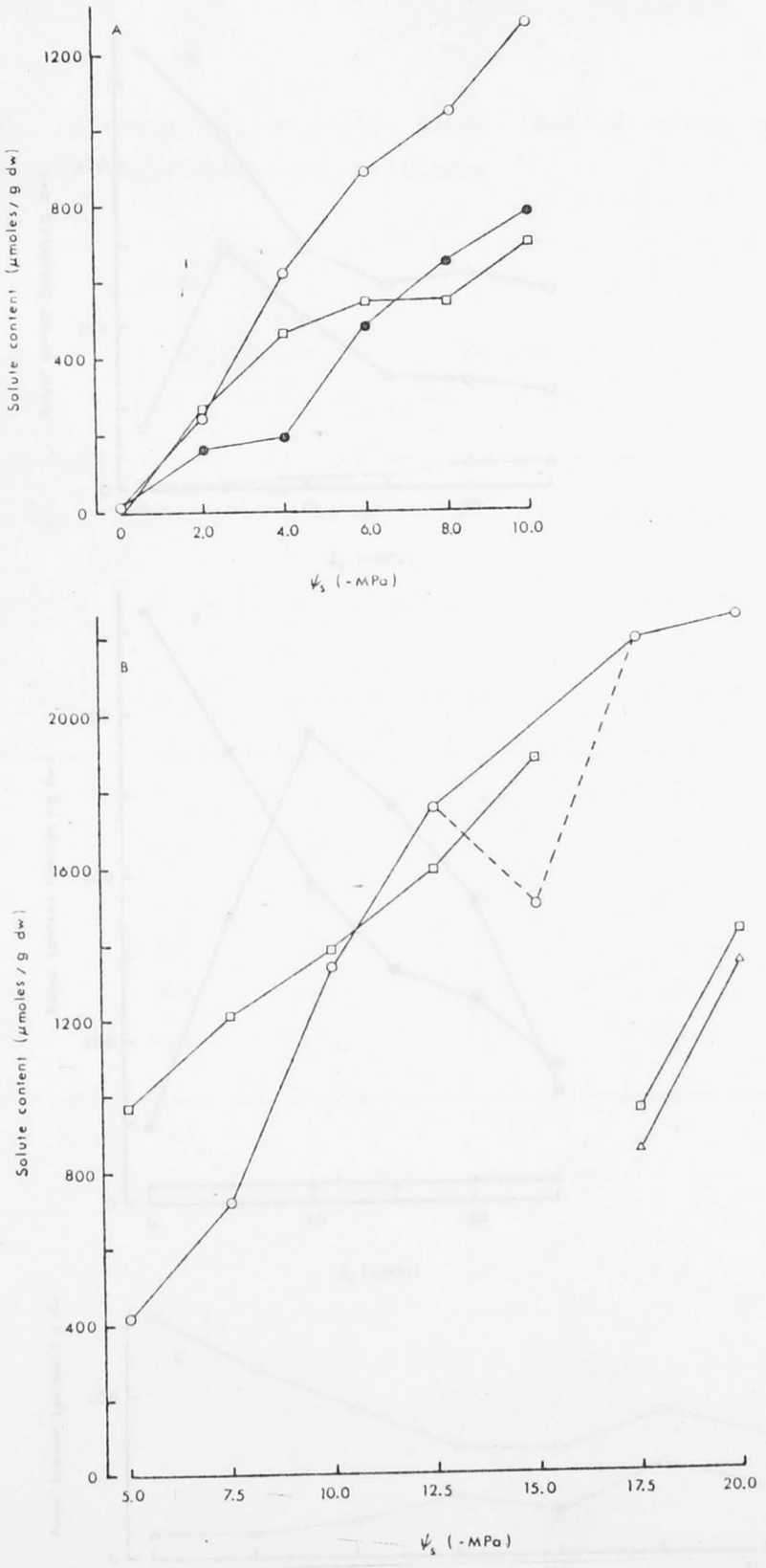


Fig. 3.4. Effect of water potential on the principal organic solutes in Penicillium chrysogenum (A) and Chrysosporium fastidium (B). Glycerol (circles), glucose (squares) and fructose (triangles). Glucose osmoticum (open symbols), KCl osmoticum (shaded symbols).

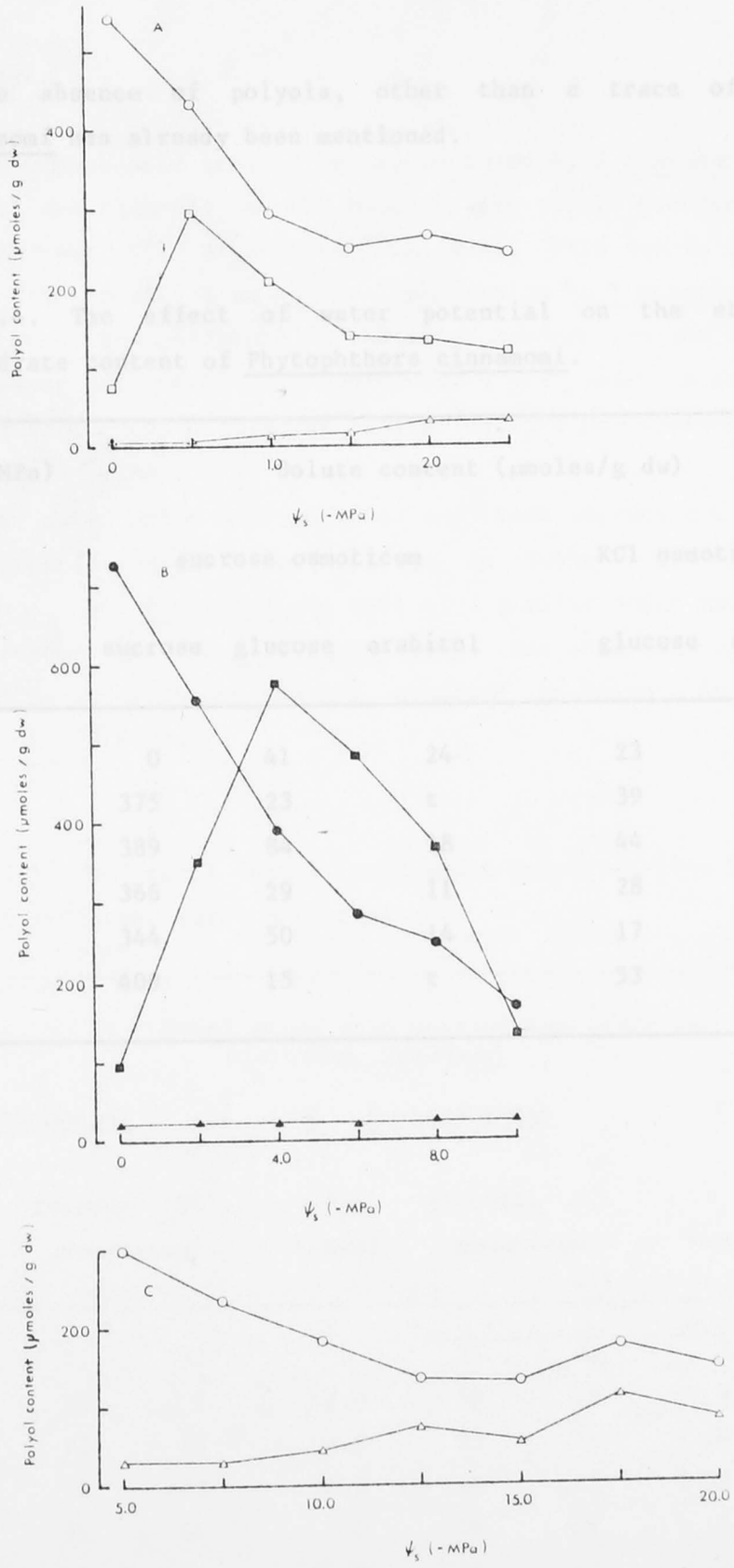


Fig. 3.5. Effect of water potential on the content of higher polyols in Penicillium chrysogenum grown on glucose (A) and on KCl (B) and in Chrysosporium fastidium grown on glucose (C). Mannitol (circles), arabitol (triangles) and erythritol (squares).

The absence of polyols, other than a trace of arabitol, in P.cinnamomi has already been mentioned.

Table 3.4. The effect of water potential on the ethanol soluble carbohydrate content of Phytophthora cinnamomi.

ψ_s (-MPa)	Solute content (μmoles/g dw)				
	sucrose osmoticum			KCl osmoticum	
	sucrose	glucose	arabitol	glucose	arabitol
0	0	41	24	23	t
0.5	375	23	t	39	11
1.0	389	84	18	44	13
1.5	366	29	11	28	t
2.0	344	50	14	17	t
2.5	409	15	t	53	12

Amino acids

The imino acid proline was accumulated by P.cinnamomi as the water potential was reduced, and the amounts were almost identical whether the osmoticum was KCl or sucrose (Fig. 3.6). This was in contrast to the solute effect observed on glycerol production by P.chrysogenum. Proline levels were insignificant in the other two species and did not respond to low water potential. CYA contained 5 g/l yeast hydrolysate which provided about 0.52 millimolar proline or 15.6 μmole/plate.

The total amino acid pools of the three species are related to the water potential of the growth medium in Table 3.5. The levels in P.cinnamomi and P.chrysogenum were of a similar order and at least four times greater than the C.fastidium pool. The addition of fructose to the medium at -17.5 and -20 MPa increased the amino acid pool as it did the polyols (Fig. 3.5).

Table 3.5 The total amino acid pools extracted from hyphae grown on media of different water potentials.

Total amino acid content (μmoles/g dw) excluding proline							
P.cinnamomi			P.chrysogenum			C.fastidium	
ψ_s (-MPa)	sucrose osmoticum	KCl	ψ_s (-MPa)	glucose osmoticum	KCl	ψ_s (-MPa)	glucose osmoticum
0	71	83	0	55	93	5.0	20
0.5	54	79	2.0	60	78	7.5	16
1.0	73	88	4.0	63	66	10.0	14
1.5	56	83	6.0	65	89	12.5	11
2.0	85	64	8.0	69	61	15.0	12
2.5	65	77	10.0	113	82	17.5	28
						20.0	26

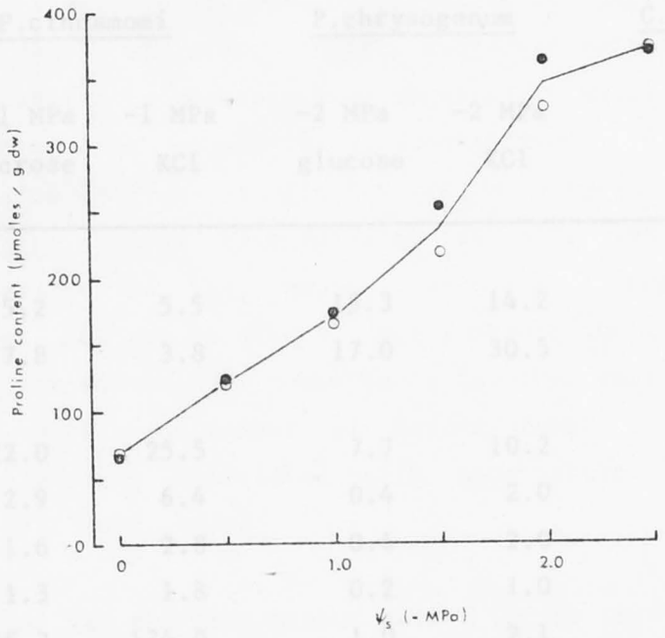


Fig. 3.6. Effect of water potential on the proline content of Phytophthora cinnamomi grown on sucrose osmoticum (open circles) and on KCl osmoticum (shaded circles).

The composition of the amino acid pools in each of the species growing at its maximum observed radial growth rate is shown in Table 3.6. The high levels of the negatively charged glutamate and aspartate in P.chrysogenum should be noted, although they did not appear to be correlated with water potential in the KCl series as might have been expected. However glutamate did increase linearly in the glucose series; -10 MPa glucose hyphae contained ten times the amount present in 0 MPa glucose material. Glutamate and aspartate were also the chief components of the pool in C.fastidium. The enhancement of the pool in the presence of fructose appeared to be the result of increased levels of almost all the pool constituents and not to any particular pathway. Other than proline, glycine and alanine were the principal pool components in P.cinnamomi.

Table 3.6. The composition of the amino acid pools extracted from hyphae exhibiting maximum observed radial growth rates.

Amino acid	Amino acid content (μmoles/g dw)				
	P.cinnamomi		P.chrysogenum		C.fastidium
	-1 MPa sucrose	-1 MPa KCl	-2 MPa glucose	-2 MPa KCl	-10 MPa glucose
Asp	5.2	5.5	15.3	14.2	2.9
Glu	7.8	3.8	17.0	30.5	4.9
Ala	22.0	25.5	7.7	10.2	2.1
Val	2.9	6.4	0.4	2.0	0.2
Leu	1.6	2.8	0.4	2.0	0.2
Ile	1.3	1.8	0.2	1.0	0.1
Pro	165.7	174.0	1.0	2.1	0.4
Phe	0.2	1.5	0.2	0.8	0.3
½Cys	0.7	1.1	0.1	0.2	0
Met	1.1	3.5	0	0	0.2
Gly	6.9	8.5	3.7	2.0	0.5
Ser	6.4	5.4	8.5	6.2	1.2
Thr	2.0	2.8	5.0	3.5	0.6
Tyr	2.0	1.8	0.2	1.0	0.2
Lys	7.5	11.0	0.4	1.1	0.3
Arg	4.0	5.0	0	0.7	0.4
His	1.2	2.0	0.2	0.9	0.1

DISCUSSION

Ions

The principal metallic elements required by fungi are potassium and magnesium; traces of zinc, copper, manganese and molybdenum are also essential. Calcium is probably also required in small amounts although this has not been conclusively demonstrated. A specific growth requirement for sodium is limited to a single report; that of Thraustochytrium roseum (Siegenthaler et al., 1967). Sodium has an inhibitory effect on the growth of some fungi, particularly Basidiomycetes, but not on others. For instance the fungi isolated from salt marshes are the same as those found in other soils (e.g. Moustafa, 1975). The NaCl requirement of marine fungi is attributed to a secondary adaptation associated with sporulation and germination (Jennings, 1973; Amon, 1976).

Potassium was the predominant cation in the three species analysed here as it is in almost all living cells, although fungi and bacteria generally have a low passive permeability to K^+ (5% of the flux) compared to fresh water algae and animal cells (30% to 75%) (Slayman, 1970). Its precise role however is not clear. Early work has shown that potassium is closely linked with carbohydrate metabolism; Aspergillus niger accumulated oxalic acid and Neurospora crassa pyruvate, under conditions of potassium deficiency (Cochrane, 1958). Both Schultz and Solomon (1961) working on Escherichia coli and Slayman and Tatum (1964) on N.crassa attributed the high K^+ content of these cells to the selective permeability of the membrane and not to binding by macromolecules.

Christian and Waltho (1961) first related the potassium content of bacteria grown in a basal medium to their tolerance of low water potential. In the 32 strains they examined, there was a positive correlation between the initial K^+ content in dilute media and the lowest potential supporting growth. It appears that this criterion also applies to P.chrysogenum and P.cinnamomi, the K^+ content of the former being 1.4 times the latter when grown on CYA without added solute, while the minimum potentials for growth were about -30 and -4 MPa

respectively.

Christian and Waltho (1961) also observed positive correlations between the K^+:Na^+ ratio and minimum potential permitting growth in gram-positive cocci and gram-negative rods, although this was not true for Bacillus species. Norkrans and Kylin (1969) compared the K^+:Na^+ ratios of a salt tolerant and a less tolerant yeast, and also found that this ratio was correlated with halotolerance. Osmophilic or sugar tolerant yeasts could be separated into two groups on the basis of their tolerance of NaCl, but not KCl or $MgCl_2$ (Rodriguez-Navarro, 1971). The K^+:Na^+ ratios of the sodium sensitive strains were lower than those of the sodium tolerant strains when grown on 0.1 molar NaCl, although at the highest sodium concentration tolerated, the ratio was greater in the sodium sensitive strain. In this case it appears that inhibition cannot be simply interpreted as an inability to maintain an adequate K^+:Na^+ ratio.

In my experimental species, the absolute value of the K^+:Na^+ ratio appeared to be related to ability to withstand osmotic stress. An ability to exclude sodium at low water potentials also distinguished C.fastidium from the other two species. This was also found to be the case in the halotolerant and non-tolerant yeasts (Norkrans & Kylin, 1969). Neither Lulworthia medusa, a marine fungus, or Ophiobolus cariceti, a freshwater isolate, were able to exclude sodium when growing in sea water, but both accumulated potassium (Davidson,1971). The K^+:Na^+ ratio of the former was about 0.6; Galpin et al. (1978) report a maximum value of 4.3 in Dendryphiella salina and Jennings (1979) has calculated values as high as 16.5 for N.crassa from the data of Slayman and Tatum (1964).

The correlation observed between the K^+ content and the growth rate of Candida utilis has prompted Aiking et al. (1977) to suggest that K^+ is closely linked with ATP synthesis. In addition, they thought that there was a small requirement for K^+ in C.utilis for osmoregulation or anion cotransport. Jennings and his group also postulated that cations can influence growth by contributing to the internal potential, particularly in conditions of low glucose when the presence of ions would free the glucose from synthesis of osmoregulators to dry weight production in

D.salina (Allaway & Jennings, 1971). This would explain the stimulation of growth observed in this species at low sodium concentrations. K^+ clearly contributed significantly to the internal potential of all three of my experimental species (Table 3.14).

Potassium is also known to accumulate and contribute to the internal potential in some bacteria and algae (e.g. Epstein & Schultz, 1965; Liu & Hellebust, 1976a). Potasssium flux has been directly related to turgor in the giant alga Valonia (Gutnecht, 1968). Zimmermann and Steudle (1978) have postulated that the K^+ flux depends on the elastic properties of the cell wall, and that the latter may regulate salt uptake and extrusion. The apparent requirement for high internal K^+ concentrations is at odds with the inhibitory effect of this cation (as well as sodium) on many in vitro enzyme systems (Chapter 4).

The chloride contents of the three experimental species were generally low when grown on sugar media (Table 3.1, 3.2 & 3.3), and the permeability of other fungi to the chloride ion also appears to be low (Shere & Jacobson, 1970). Kirst (1977) summarised the literature on the ion content of algae and concluded that the Cl^- content of non-vacuolate microalgae was very low compared with the vacuolate giant and thalloid algae. This appears to agree with the situation in fungi which are also largely non-vacuolate. The nature of the balancing anions in microalgae was unknown. The possible importance of organic acid anions in fungi has already been mentioned. Bicarbonate and phosphate were the principal anions in resting cells of Saccharomyces cerevisiae (Rothstein, 1960).

No obvious changes in hyphal content of magnesium or calcium resulted from the alteration of external potential (Tables 3.1, 3.2 & 3.3). The principal essential function of magnesium is in the activation of enzymes, although none of the symptoms of magnesium deficiency have been directly linked to enzyme malfunction. The molar stoichiometry between Mg^{2+} and RNA established in prokaryotes was not demonstrated in Aspergillus nidulans (McGetrick & Bull, 1979). Calcium has an effect on the growth of fungi; it stimulates dry weight production in many but not all species over a wide range of

concentrations. The levels of Mg^{2+} and Ca^{2+} involved in these requirements is low and was not limited in the cultural conditions used in the present study.

Divalent cations also appear to have an ability to limit the increase in permeability and subsequent loss of solutes which may result from an excess of sodium (Jones & Jennings, 1965; Allaway & Jennings, 1970a; 1970b; 1971). The high salt tolerance of many fungi, which is particularly obvious in the Aspergillus and Penicillium group (Tresner & Hayes, 1971), may be due to the independence of the glucose uptake mechanism from the internal K^+ concentration, or to a resistance to Na^+ induced permeability changes. The inability of C.fastidium to tolerate salt in my experiments may therefore be due to a difference in membrane characteristics with respect to ions.

Carbohydrate

Glycerol was accumulated in large amounts by both P.chrysogenum and C.fastidium as an osmoregulator, presumably synthesised from triose phosphate which is an early metabolite in the major catabolic route for glucose found in microorganisms; the Emden-Meyerhof-Parnas scheme of glycolysis. Cleavage of fructose-1-6-diphosphate by aldolase produces a mixture of dihydroxyacetone phosphate and glyceraldehyde phosphate. The latter proceeds through the glycolytic pathway to pyruvate. Under anaerobic conditions in yeasts and a few filamentous fungi, pyruvate may be decarboxylated to acetaldehyde and then reduced to ethanol. When conditions favour glycerol production, dihydroxyacetone phosphate is reduced and then dephosphorylated to glycerol. The reduction step requires NADH which is then not available to reduce acetaldehyde. Two conditions favouring the formation of glycerol rather than ethanol are alkaline pH and the presence of sulphite. The diagram of Mahler and Cordes (1971) is reproduced with some additions and shows the positions of ethanol and glycerol in relation to the main glycolytic pathway (Fig. 3.7).

Triose phosphate also occurs as an intermediate in the pentose phosphate pathway. Lowe and Jennings (1975) have accounted for label in

arabitol originating from glucose as proceeding via glycerol in D.salina although symmetry in the glycerol and mannitol molecules results in randomization of specifically labelled carbon atoms, making interpretation of sequential labelling experiments difficult.

NADH dependent triose phosphate reducing activity and not alcohol dehydrogenase in S.cerevisiae approximately accounted for the glycerol production in this species (Gancedo et al., 1968). The enzyme apparently required low ionic strength for maximum activity, and the concentration in yeast was greatest when the carbon source was hexose. Two groups have independently shown the presence of an NADPH specific glycerol dehydrogenase in species of the marine alga Dunaliella (Ben-Amotz & Avron, 1973; Borowitzka & Brown, 1974). This enzyme catalyzes the direct reduction of dihydroxyacetone to glycerol so that dephosphorylation precedes the reduction in this group, in contrast to the scheme occurring in yeast (Fig. 3.7).

It is likely that the site of regulation of glycerol accumulation is close to glycerol or even at glycerol itself (Brown, 1978). Brown has hypothesized that regulation may be mediated by alteration of the ratio of oxidised to reduced coenzyme which in turn might be associated with energy linked proton fluxes (Slayman, 1977). Also the requirement for reduced coenzyme and hence ATP for glycerol production has prompted Brown to suggest that ATP:ADP ratios may be of central importance in regulation. Gustafsson (1979) has indeed found that the ATP concentration in the marine yeast Debaryomyces hansenii increased at high levels of salinity.

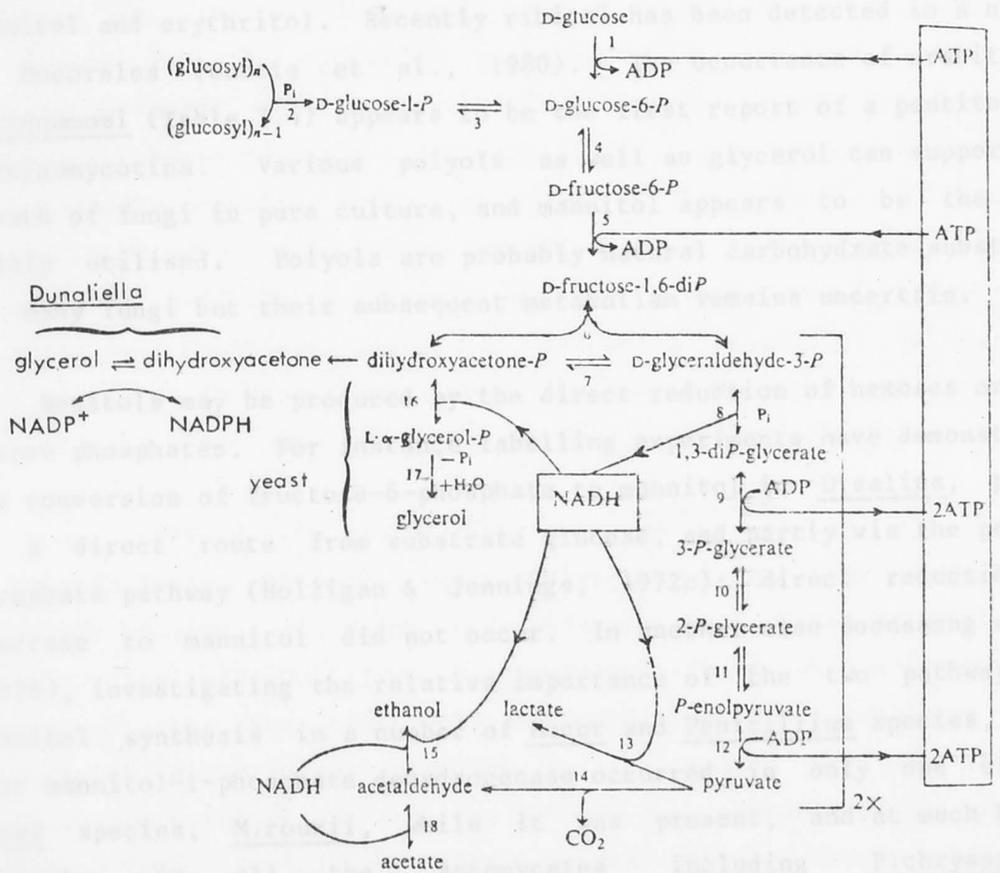


Fig. 3.7. The Embden-Meyerhof-Parnas scheme of glycolysis (After Mahler and Cordes, 1971).

Lewis and Smith (1967) have reviewed the information concerning higher polyols in fungi and noted their widespread occurrence, although they appear to be absent from aseptate fungi. Ribitol and glucitol have been identified subsequently as major components of mycelium, but not spores, of the wheat stem rust fungus Puccinia graminis (Maclean & Scott, 1976). These compounds have not previously been detected in fungi and occurred in addition to the more common polyols, arabitol, mannitol and erythritol. Recently ribitol has been detected in a number of Mucorales (Curtis et al., 1980). The occurrence of arabitol in P.cinnamomi (Table 3.4) appears to be the first report of a pentitol in Mastigomycotina. Various polyols as well as glycerol can support the growth of fungi in pure culture, and mannitol appears to be the most widely utilised. Polyols are probably natural carbohydrate substrates for many fungi but their subsequent metabolism remains uncertain.

Hexitols may be produced by the direct reduction of hexoses or from hexose phosphates. For instance labelling experiments have demonstrated the conversion of fructose-6-phosphate to mannitol in D.salina, partly in a direct route from substrate glucose, and partly via the pentose phosphate pathway (Holligan & Jennings, 1972c); direct reduction of fructose to mannitol did not occur. In another case Boonsaeng et al. (1976), investigating the relative importance of the two pathways of mannitol synthesis in a number of Mucor and Penicillium species, found that mannitol-1-phosphate dehydrogenase occurred in only one of the Mucor species, M.rouxii, while it was present, and at much higher activity, in all the Ascomycetes including P.chrysogenum. Mannitol-1-phosphatase activity was also demonstrated in P.notatum, completing the pathway. As in D.salina, no direct reduction of fructose could be detected in P.notatum. Mannitol-1-phosphatase has also been demonstrated in a number of other species, but several yeasts and Basidiomycetes have been shown to reduce fructose to mannitol directly (Lewis & Smith, 1967).

Levels of phosphofructokinase, the enzyme which might regulate the relative proportions of fructose-6-phosphate proceeding to glycolysis or mannitol synthesis, proved difficult to measure in both Moniliella tomentosa, a yeast like fungus producing large amounts of glycerol and erythritol and in Ascomycetes (Hanssens et al., 1974; Boonsaeng et al.,

1976). If this was the result of low levels, it might indicate the importance of the pentose phosphate pathway relative to glycolysis in xerotolerant organisms and account for lower growth rates (Brown, 1978).

Pentitols are formed only by the direct reduction of pentoses which are intermediates in the pentose phosphate pathway. The current view of the pentose phosphate pathway is that it is unlikely to constitute a sequence for the breakdown of glucose or to act as a cycle, but rather it provides for the oxidative conversion of glucose-6-phosphate to pentose phosphate, and the anaerobic synthesis of pentose phosphate from fructose-6-phosphate (Lowe & Jennings, 1975). The products are pentoses and reduced coenzyme which are used for biosynthesis. The pentose phosphate pathway has long been demonstrated on P.chrysogenum (Sih et al., 1957) but its existence must be presumed in P.cinnamomi and C.fastidium. Doubtless tetritols, including erythritol produced by P.chrysogenum in these experiments, are formed by such a mechanism from tetroses which are also intermediates in the pentose phosphate pathway.

The specificity of the enzymes involved, the polyol oxido-reductases, appears to vary. Chiang and Knight (1959) isolated an inducible enzyme catalysing the reduction of xylose to xylitol and arabinose to arabitol from P.chrysogenum grown on xylose; they considered that the equilibrium favoured aldopentose reduction. They also showed that arabinose provided in the medium was converted to ribulose and xylulose via arabitol in cell free extracts of this species (Chiang & Knight, 1961). An NADPH dependent polyol oxido-reductase has been isolated from the osmophilic yeast Pichia miso which was non-specific and able to utilize glycerol, erythritol, xylitol, ribitol, D-arabitol, D-glucitol, galactitol and D-mannitol with the production of ketoses (Onishi & Saito, 1962). Ingram and Wood (1965) also considered that a non-specific phosphatase and polyol dehydrogenase were responsible for derivation of polyols from intermediates of the pentose phoshate pathway in S.rouxii; arabitol was produced directly from ribulose. Labelling experiments in D.salina suggested that arabitol synthesis from ribose was via ribulose and xylulose (Lowe & Jennings, 1975). Burnett (1976) has produced a diagram summarizing these pathways

which has been reproduced in Fig. 3.8 with the addition of the information on Penicillium.

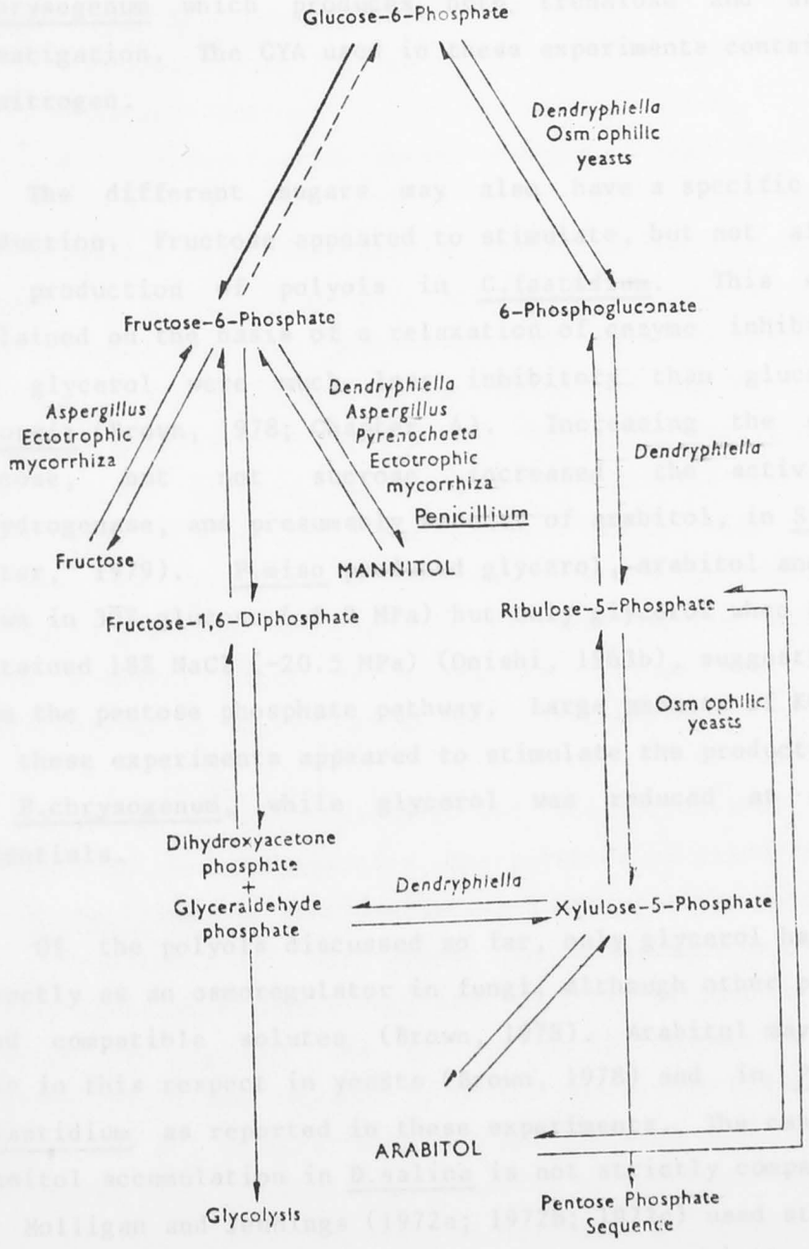


Fig. 3.8. Principal pathways of polyol biosynthesis in fungi. (After Burnett, 1976).

Lewis and Smith (1967) have emphasised the effect of growth conditions on the relative production of different polyols by the same fungus. For instance growth of D.salina on inorganic nitrogen rather than amino acids increased arabitol but not mannitol production and this was attributed to a stimulation of the pentose phosphate pathway (Holligan & Jennings, 1972a; 1972b). Similarily, inorganic nitrogen favoured trehalose and reduced mannitol synthesis in Claviceps purpurea (Vining & Taber, 1964). The effect of different nitrogen sources in P.chrysogenum which produces both trehalose and arabitol requires investigation. The CYA used in these experiments contained both forms of nitrogen.

The different sugars may also have a specific effect on polyol production. Fructose appeared to stimulate, but not alter relatively, the production of polyols in C.fastidium. This can probably be explained on the basis of a relaxation of enzyme inhibition. Fructose and glycerol were much less inhibitory than glucose to enzymes of S.rouxii (Brown, 1978; Chapter 4). Increasing the concentration of glucose, but not sucrose increased the activity of arabitol dehydrogenase, and presumably amounts of arabitol, in S.rouxii (Moran & Witter, 1979). P.miso produced glycerol, arabitol and erythritol when grown in 30% glucose (-6.2 MPa) but only glycerol when the growth medium contained 18% NaCl (-20.5 MPa) (Onishi, 1963b), suggesting a switch away from the pentose phosphate pathway. Large amounts of KCl in the medium in these experiments appeared to stimulate the production of erythritol in P.chrysogenum, while glycerol was reduced at comparable water potentials.

Of the polyols discussed so far, only glycerol has been implicated directly as an osmoregulator in fungi, although other polyols are also good compatible solutes (Brown, 1978). Arabitol may play a secondary role in this respect in yeasts (Brown, 1978) and in P.chrysogenum and C.fastidium as reported in these experiments. The case of arabitol and mannitol accumulation in D.salina is not strictly comparable. The work of Holligan and Jennings (1972a; 1972b; 1972c) used starved cultures of stationary phase cells; the shock experiments of Allaway and Jennings (1970a; 1970b; 1971) were followed for only 5 hours and the lowest potential in both cases was greater than -1 MPa. Glycerol was in fact

detected in some of their experiments but not in significant quantities. However polyols and polyol derivatives do appear to have an osmoregulatory function in some algae; for instance cyclohexane tetrol increased in Monochrysis lutheri (Craigie, 1969), isofloridoside (galactosyl glycerol) in Ochromonas malhamensis (Kauss, 1973) and mannitol in Platymonas subcordiformis (Kirst, 1977).

Lewis and Smith (1967) have suggested possible roles for higher polyols other than osmoregulation:

1. carbohydrate storage
2. translocation
3. coenzyme regulation and storage of reducing power.

There has been considerable discussion on the interrelationships between the levels of polyols, particularly mannitol, with disaccharides such as trehalose and polysaccharides such as glycogen, and their importance as storage products. They have long been assumed to fulfil this role particularly because they have often been implicated in spore germination. However this last involvement is still not clear (Lewis & Smith, 1967). Rast (1965) has analysed the fruiting body of Agaricus bisporus to attempt to clarify these interrelationships, but she found no simple gradients between stem, cap and lamella and concluded that while mannitol might be acting as a translocatory carbohydrate, she could suggest no physiological role for trehalose.

Although oligosaccharides and polysaccharides have not been analysed in these experiments, the presence of glycogen in P.chrysogenum and C.fastidium has been demonstrated by electron microscopy (Chapter 5). Trehalose did not appear to be a major pool constituent. The steady state experiments suggested that the pattern of mannitol was the inverse of glycerol and glucose or KCl content (Fig. 3.4 & 3.5). A similar result was obtained by Onishi and Suzuki (1968) for the effects of NaCl and KCl on three mannitol and glycerol producing yeasts. It was considered that conversion of mannitol to insoluble polysaccharide was part of the osmoregulatory mechanism in D.salina (Jennings & Austin, 1973; McDermott & Jennings, 1976), and it is likely that such a mechanism also operated in P.chrysogenum and C.fastidium.

In summary, glycerol is probably the primary osmoregulator in P.chrysogenum and C.fastidium while arabitol may be a minor osmoregulator. It might be deduced that mannitol is acting as carbohydrate reserve for glycerol synthesis or an energy reserve which is released for glycerol synthesis, or glucose or KCl uptake. Erythritol may well be a reserve of reducing power, since it appeared to be correlated with the growth rate.

Amino acids

Proline increase in response to water stress has been recorded in higher plants, particularly the Chenopodiaceae and Graminae (e.g. Chu et al., 1976; Storey & Wyn Jones, 1977); in bacteria (e.g. Britten & McClure, 1962; Christian & Hall, 1972; Measures, 1975) and algae (e.g. Liu & Hellebust, 1976a; Brown & Hellebust, 1978) but there is no previous record of its accumulation in fungi. The green alga Stichococcus bacillaris is of interest in that it has been shown to accumulate both a polyol (sorbitol) and an amino acid (proline) (Brown & Hellebust, 1978). In microorganisms ammonia from inorganic nitrogen sources is initially converted to an organic form as glutamate, and proline is formed from glutamate via glutamic semi-aldehyde and is therefore an early product of nitrogen metabolism.

Other ammonium compounds may also act as osmoregulators. Glutamic acid and γ-amino butyric acid are important in some non-halophilic bacteria (Measures, 1975). Storey et al. (1977) have established that the quarternary ammonium compound glycine betaine is correlated with salt resistance and is characteristically found in the Graminae and Chenopodiaceae in addition to proline. The fungal species of the present study were not analysed for quarternary ammonium compounds and it would be of interest to determine whether they also occur in P.cinnamomi.

Proline was accumulated by the bacterium Salmonella orianenburg and its presence in the medium was essential for growth below 0.97 a_w (-4.2 MPa) (Christian, 1955a). Proline increased the ability of this organism to accumulate K^+ as the water potential decreased (Christian & Hall, 1972) and uptake of γ-amino butyric acid and K^+ were also

correlated in a marine pseudomonad (Thompson & MacLeod, 1974). There was no evidence of K^+ correlation with proline accumulation in P.cinnamomi in terms of final contents (Table 3.1) although rates of uptake were not investigated. In both Staphylococcus aureus (Christian & Waltho, 1964; Koujima et al., 1978) and S.orianenburg (Christian & Hall, 1972), the K^+ pool was greater than the total proline pool at higher water potentials, but the latter became the predominant solute at the lower water potentials. In P.cinnamomi the relative contributions of the two solutes depended on the external osmoticum (Table 3.14).

Measures (1975) has divided the responses of the non-halophilic bacteria to water stress into the gram-negative species which have low amino acid pools composed mainly of glutamate and also have low K^+ levels, and the gram-positives which have large free amino acid pools under unstressed conditions and also high K^+ levels. The latter may contain limiting concentrations of glutamate, the further accumulation of which would require the balancing charge of inhibitory cations. He therefore suggests that a mechanism has developed for the formation of the neutral amino acid proline in the gram-positive species. P.chrysogenum and C.fastidium appear to resemble the gram-negative bacteria in terms of size and composition of the amino acid pool, and P.cinnamomi the gram-positives. Unemoto & Hayashi (1979) also found that in contrast to the non-halophilic bacteria and the extreme halophiles, amino acids and not K^+ were the principal osmoregulatory solutes in the moderately halophilic Vibrio alginolyticus.

The importance of proline as an osmoregulator does not appear to be entirely unequivocal. Cavalieri and Huang (1979) sub-divided the responses of salt marsh plants on the basis of the threshold salinity which stimulated proline accumulation and concluded that this compound was important in only some of the species within this group. Proline accumulation did not occur at all in one species, Plantago maritima (Stewart & Lee, 1974). 'Resurrection' plants are a group which exhibit the extreme of higher plant tolerance to drought stress; they are able to survive dehydration to air dryness and may have leaf water potentials as low as 7% r.h. (<-100 MPa). Tymms and Gaff (1979) have used this group of plants to investigate the role of proline in water stress. They concluded that although proline did accumulate in some species

during the drying cycle, it was more likely to be associated with loss of chlorophyll on drying and did not account for 'resurrection'. A survey of the lower fungi would be useful to determine whether or not the accumulation of proline is a general phenomenon in this group.

The total amino acid pool as distinct from osmoregulatory amino acids, seems to show no predictable pattern in response to water stress. Given that stress generally reduces growth rate, the simplest assumption is that the amino acid pool should decrease with low water potential. This was clearly the case only in C.fastidium. Tempest et al. (1970) did report some variation in the size and composition of the total pool in some bacteria and yeast, dependent on the growth rate, but this was small compared to the osmoregulatory increase in glutamate in the bacteria. The total amino acid content of V.alginolyticus increased from 0.036 to 0.87 molar as the medium NaCl concentration increased from 0.2 to 1.5 molar (-0.9 to -7.3 MPa) (Unemoto & Hayashi, 1979). In the halotolerant yeast D.hansenii, the total content of amino acids was 15 to 50% lower in cells cultured in 2.7 molar NaCl (-14.6 MPa) than in cells cultured in 0.004 molar. The total pool of amino acids other than proline remained relatively constant in S.bacillaris (Brown & Hellebust, 1978), while cells of the brackish water diatom C.cryptica grown in 80% artificial sea water (-2.2 MPa) had a higher total amino acid content than those grown in 33% sea water (-0.9 MPa) (Liu & Hellebust, 1976a).

THE EFFECT OF OSMOTIC SHOCK ON HYPHAL SOLUTES

An osmotic shock was applied simply by lifting the cellophane with the colony from the surface of the agar on which it had grown and laying it carefully on the agar of a fresh plate of the desired potential. The procedure took a few seconds only.

Phytophthora cinnamomi was grown on 0 MPa KCl and -2 MPa KCl until the colonies reached a radius of 20 mm; 5 days in the former and 10 days in the latter. Each treatment was then subjected to hyperosmotic or hypoosmotic shock; colonies grown on 0 MPa were transferred to -2 MPa KCl plates and vice versa. Colonies were analysed for cations, chloride and amino acids for up to 8 h after the transfer. The change in hyphal osmotic potential was also followed, including the first few minutes after the transfer.

Penicillium chrysogenum colonies were grown on 0 MPa glucose and -10 MPa glucose until they had reached a radius of 20 mm; 6 days in the former, 12 days in the latter. Again the colonies were subjected to shock by transferring to fresh -10 MPa glucose or 0 MPa plates accordingly. Samples were taken for up to 8 h after the transfer for analyses of cations, chloride, carbohydrate and amino acids. Osmotic potentials of the hyphae were measured for 24 h after the transfer.

In an attempt to explain the obligate xerophily of Chrysosporium fastidium and its intolerance of salt, this species was subjected to hypoosmotic shock and to isoosmotic KCl, conditions in which it will not normally grow. Colonies were grown on -10 MPa glucose for 17 days (20 mm radius) and then transferred to 0 MPa or to -10 MPa KCl. Cations, chloride and carbohydrate were analysed for 24 h after the transfer, and osmotic potentials of the hyphae were obtained.

RESULTS

Hyphal osmotic potential

The hyphal osmotic potentials following hyperosmotic and hypoosmotic shock are shown in Fig. 3.9. The rate of change was extremely rapid and, in the latter, was virtually complete in a matter of minutes. This rapid time course is indicative of passive water loss or gain rather than energy dependent solute adjustment. The change in fresh weight to dry weight ratios supported this assumption (Table 3.7). The time course following hyperosmotic shock was rather less rapid, most noticeably in P.chrysogenum. Water efflux, as discussed earlier, does not account for the total change in osmotic potential and the new value was not reached for about 7 h, dependent on synthesis or accumulation of solutes.

The effect of hypoosmotic shock on C.fastidium appeared to be the same as on P.chrysogenum. A positive turgor of several MPa was maintained at the high water potential for at least 24 h. Similarily, transfer to isoosmotic KCl had no effect on the osmotic potential and turgor was clearly maintained under these conditions.

Table 3.7. The fresh weight : dry weight ratios of *Phytophthora cinnamomi*, *Penicillium chrysogenum* and *Chrysosporium fastidium* following osmotic shock. See text for details of potential difference of shock.

Time (h)	Fresh weight:dry weight					
	P.cinnamomi		*P.chrysogenum*		*C.fastidium*	
	Hypo-osmotic	Hyper-osmotic	Hypo-osmotic	Hyper-osmotic	Hypo-osmotic	Iso-osmotic
0	3.97	3.95	2.30	3.43	2.98	2.98
0.5	4.87	3.00	3.22	2.45	4.15	3.31
1	5.16	2.65	3.24	2.39	4.24	3.19
2	4.61	3.31	3.32	2.29	-	-
4	5.14	3.17	3.30	2.22	4.43	3.23
8	4.90	3.61	3.23	2.14	4.15	3.30
24	-	-	-	-	3.55	3.23

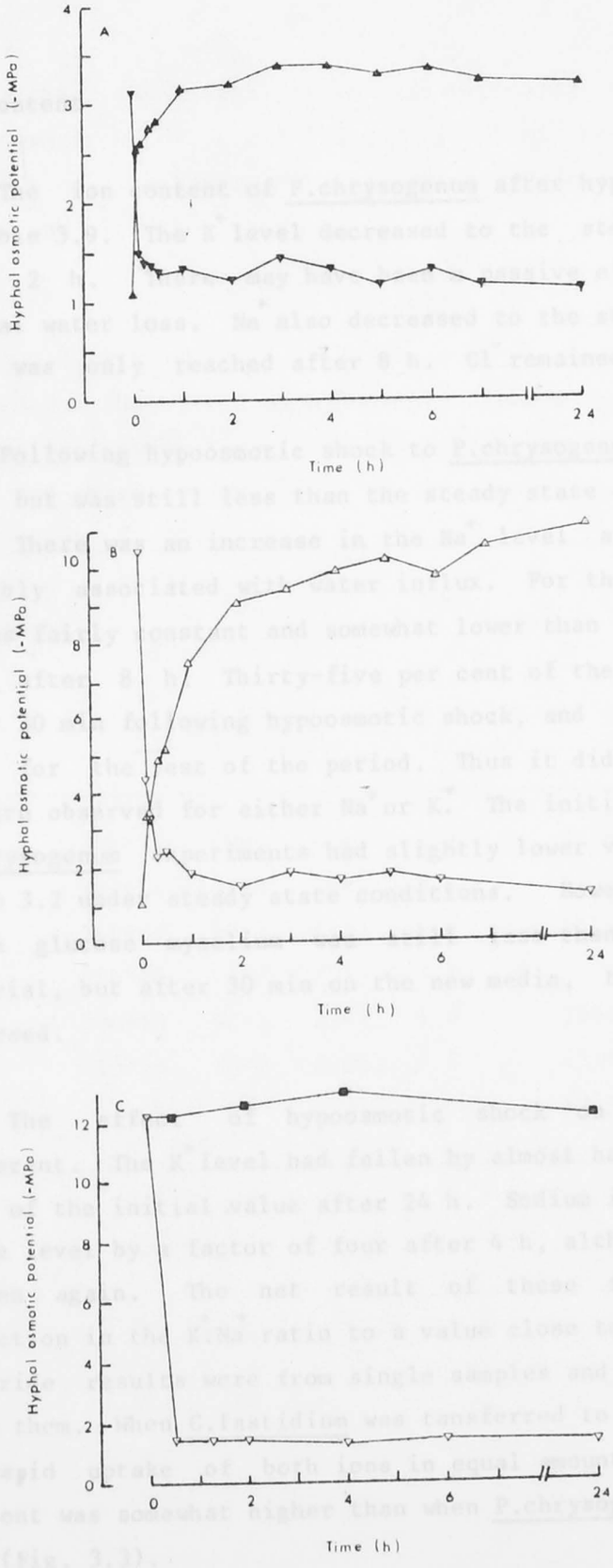


Fig. 3.9. Effect of osmotic shock on the hyphal osmotic potentials of Phytophthora cinnamomi (A), Penicillium chrysogenum (B) and Chrysosporium fastidium (C). Hypoosmotic shock (▽,▼), hyperosmotic shock (△,▲) and isoosmotic shock (■). KCl osmoticum (shaded symbols), glucose osmoticum (open symbols).

Ion content

The ion content of P.chrysogenum after hyperosmotic shock is shown in Table 3.9. The K^+ level decreased to the steady state level after about 2 h. There may have been a passive efflux associated with the initial water loss. Na^+ also decreased to the steady state level, but this was only reached after 8 h. Cl^- remained approximately constant.

Following hypoosmotic shock to P.chrysogenum, K^+ increased (Table 3.8) but was still less than the steady state content (Table 3.2) after 8 h. There was an increase in the Na^+ level after the first 30 min possibly associated with water influx. For the rest of the period, the Na^+ was fairly constant and somewhat lower than the steady state level, even after 8 h. Thirty-five per cent of the chloride was lost in the first 30 min following hypoosmotic shock, and the level continued to fall for the rest of the period. Thus it did not appear to follow the pattern observed for either Na^+ or K^+. The initial K^+:Na^+ ratios for both P.chrysogenum experiments had slightly lower values then those shown in Table 3.2 under steady state conditions. However the ratio for the 0 MPa glucose mycelium was still less than that for -10 MPa glucose material, but after 30 min on the new media, the relative values had reversed.

The effect of hypoosmotic shock on C.fastidium was rather different. The K^+ level had fallen by almost half after 30 min and was 40% of the initial value after 24 h. Sodium increased above the steady state level by a factor of four after 4 h, although after 24 h it had fallen again. The net result of these two trends was a drastic reduction in the K^+:Na^+ ratio to a value close to that of the medium. The chloride results were from single samples and little can be interpreted from them. When C.fastidium was tansferred to isoosmotic KCl there was a rapid uptake of both ions in equal amounts (Fig. 3.10). The final content was somewhat higher than when P.chrysogenum was grown on -10 MPa KCl (Fig. 3.3).

P.cinnamomi was subjected to salt shock and the subsequent changes in ion content are shown in Fig. 3.10. Equilibration following

hypoosmotic shock was rapid and virtually complete after 1 h. After hyperosmotic shock, K^+ was 76% and Cl^- 78% of the new steady state value after 8 h. The reason for the apparent decrease in both K^+ and Cl^- after 4 h is not clear.

Table 3.8. The effect of hypoosmotic shock on the ion contents of Penicillium chrysogenum and Chrysosporium fastidium. Values are the means and standard deviations of three samples.

Time (h)	K^+	Na^+	Cl^-	$K^+:Na^+$	K^+	Na^+	Cl^-	$K^+:Na^+$
	(μequiv/g dw)				(μequiv/g dw)			
	P.chrysogenum				C.fastidium			
0	346±22	70±2	52±5	4.9	317±6	38±3	60	8.3
0.5	347±14	101±8	34±3	3.4	170±4	138±4	43	1.3
1.0	361±27	90±7	31±3	4.0	191±6	128±2	35	1.5
2.0	349±28	91±7	29±3	3.8	-	-	-	-
4.0	370±13	92±3	25±1	4.0	189±3	154±5	194	1.2
8.0	413±33	88±7	27±1,	4.7	131±12	116±5	72	1.1
24.0	-	-	-	-	125±12	5±15	135	1.5

Table 3.9. The effect of hyperosmotic shock on the ion content of Penicillium chrysogenum.

Time (h)	K^+	Na^+	Cl^-	$K^+:Na^+$
	(μequiv/g dw)			
0	453±12	99±3	44±5	4.6
0.5	442±12	101±4	45±1	4.4
1.0	408±14	94±2	45±3	4.3
2.0	390±20	84±3	39±1	4.6
4.0	393±10	86±4	41±3	4.6
8.0	374±18	69±10	37±3	5.4

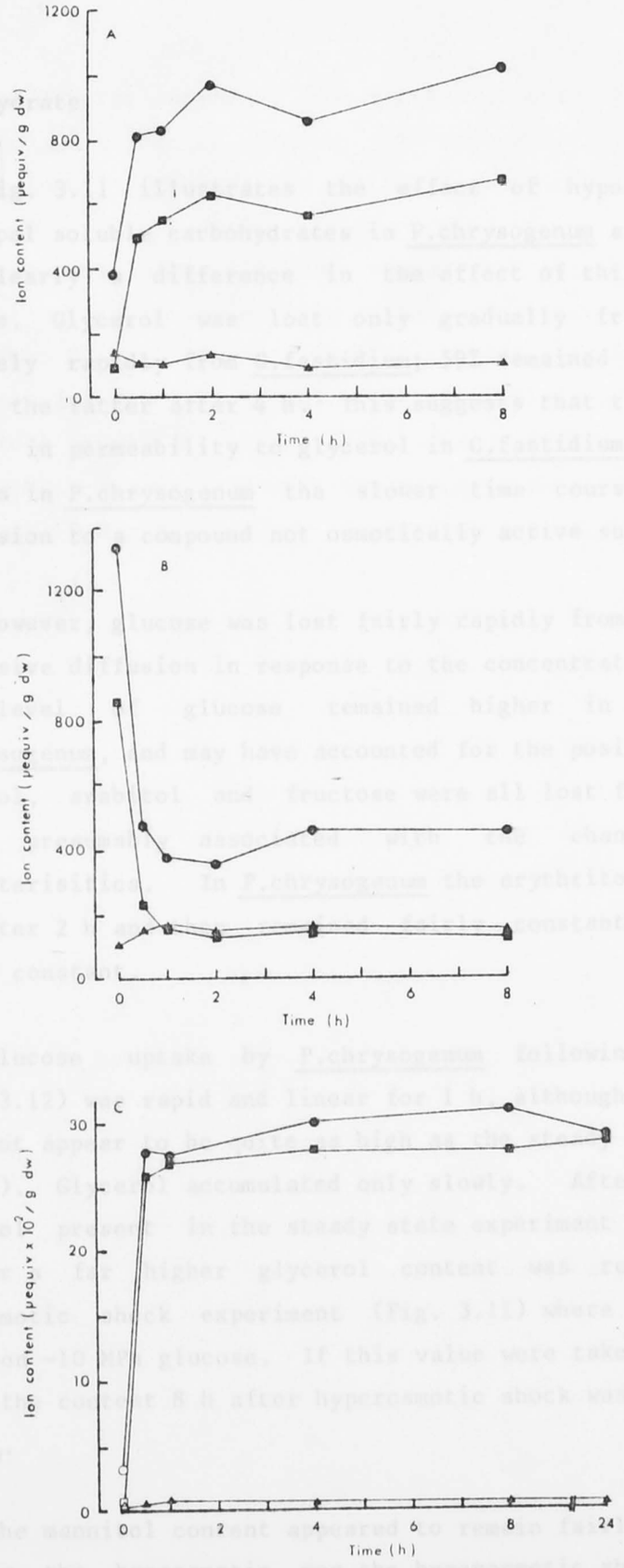


Fig. 3.10. Effect of hyperosmotic shock (A) and hypoosmotic shock (B) on the ion content of Phytophthora cinamomi and after isoosmotic transfer of Chrysosporium fastidium to KCl (C). Potassium (●), sodium(▲) and chloride (■).

Carbohydrate

Fig. 3.11 illustrates the effect of hypoosmotic shock on the principal soluble carbohydrates in P.chrysogenum and C.fastidium. There was clearly a difference in the effect of this treatment on the two species. Glycerol was lost only gradually from P.chrysogenum but extremely rapidly from C.fastidium; 59% remained in the former but only 13% in the latter after 4 h. This suggests that the shock triggered a change in permeability to glycerol in C.fastidium resulting in leakage, whereas in P.chrysogenum the slower time course suggests metabolic conversion to a compound not osmotically active such as glycogen.

However, glucose was lost fairly rapidly from both species probably by passive diffusion in response to the concentration gradient. In fact the level of glucose remained higher in C.fastidium than in P.chrysogenum, and may have accounted for the positive turgor observed. Mannitol, arabitol and fructose were all lost from C.fastidium (Table 3.10), presumably associated with the change in permeability characterisitics. In P.chrysogenum the erythritol content decreased to 23% after 2 h and then remained fairly constant. Arabitol remained fairly constant.

Glucose uptake by P.chrysogenum following hyperosmotic shock (Fig. 3.12) was rapid and linear for 1 h, although the plateau reached did not appear to be quite as high as the steady state value (Figs. 3.4 & 3.11). Glycerol accumulated only slowly. After 8 h, 95% of the glycerol present in the steady state experiment (Fig. 3.4) had formed. However a far higher glycerol content was recorded in the time$_o$ hypoosmotic shock experiment (Fig. 3.11) where the colonies were also grown on -10 MPa glucose. If this value were taken as the steady state, then the content 8 h after hyperosmotic shock was only 53% of the final amount.

The mannitol content appeared to remain fairly constant throughout. Neither the hypoosmotic nor the hyperosmotic shock treatments suggest that mannitol was implicated as an osmoregulatory compound or its precursor. The erythritol level was somewhat higher than previously measured steady state levels and also remained fairly constant.

Arabitol however increased from a trace at time$_o$ to 53 μmoles/g dw after 8 h, and as suggested before, it may well be acting as a minor osmoregulator although the actual amounts involved are insignificant.

Isoosmotic salt did not appear to alter the permeability of C.fastidium to glycerol (Fig. 3.12). At least there was only a slight loss after 24 h. Glucose was lost, 16% remaining after 24 h, in exchange for KCl (Fig. 3.10). The mannitol level was variable although after 24 h it was 30% of the initial value (Table 3.10). The appearance of erythritol coincided with a maximum in mannitol and arabitol after 4 h on the -10 MPa KCl medium. Erythritol was not detected after 24 h.

Table 3.10. The effect of shock treatment on the minor soluble carbohydrate content of *Penicillium chrysogenum* and *Chrysosporium fastidium*.

Time (h)	Carbohydrate content (μmoles/g dw)											
	P.chrysogenum						*C.fastidium*					
	Hypoosmotic			Hyperosmotic			Hypoosmotic			Isoosmotic		
	E	A	F	E	A	F	E	A	F	E	A	F
0	173	17	t	271	t	t	0	46	83	0	46	83
0.5	85	20	t	161	16	t	0	19	t	-	-	-
1.0	67	21	t	179	21	t	0	25	t	t	79	t
2.0	39	15	t	207	30	t	-	-	-	-	-	-
4.0	36	15	t	270	50	t	0	t	t	63	92	t
8.0	38	15	t	223	53	t	0	t	t	0	55	t
24.0	-	-	-	-	-	-	0	0	t	0	21	t

t = trace

E = erythritol

A = arabitol

F = fructose

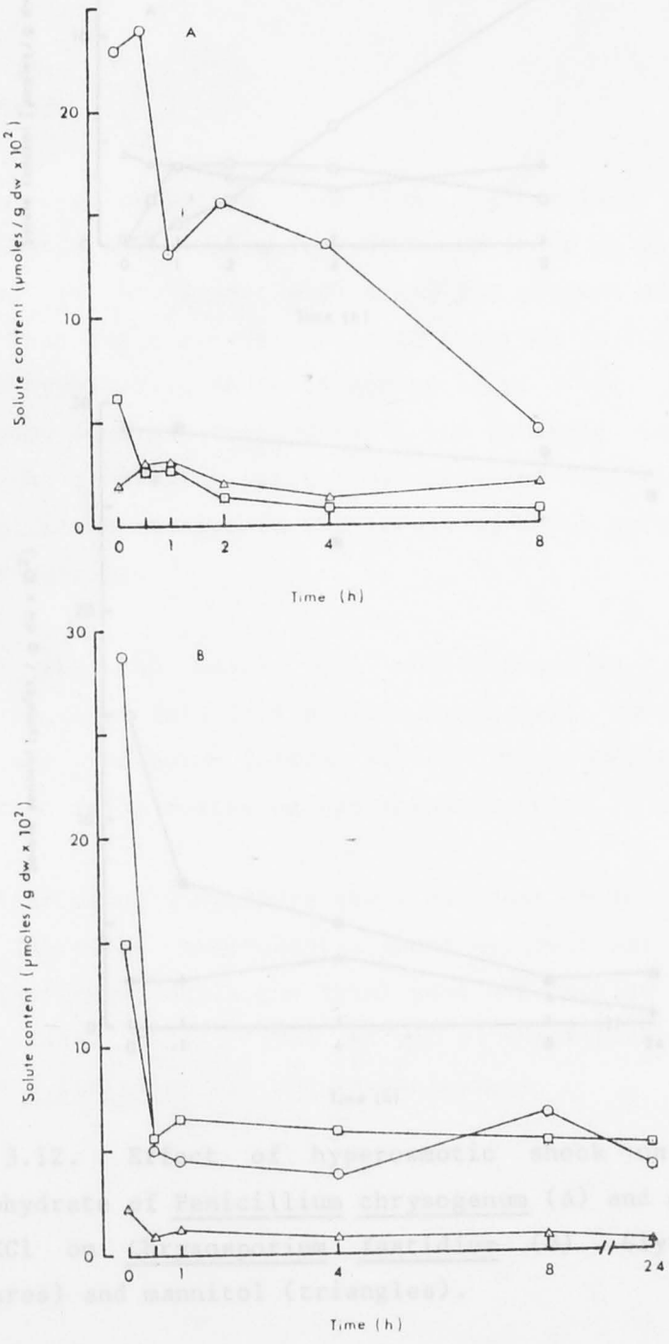


Fig. 3.11. Effect of hypoosmotic shock on the principal soluble carbohydrate content in Penicillium chrysogenum (A) and Chrysosporium fastidium (B). Glycerol (○), glucose (□) and mannitol (△).

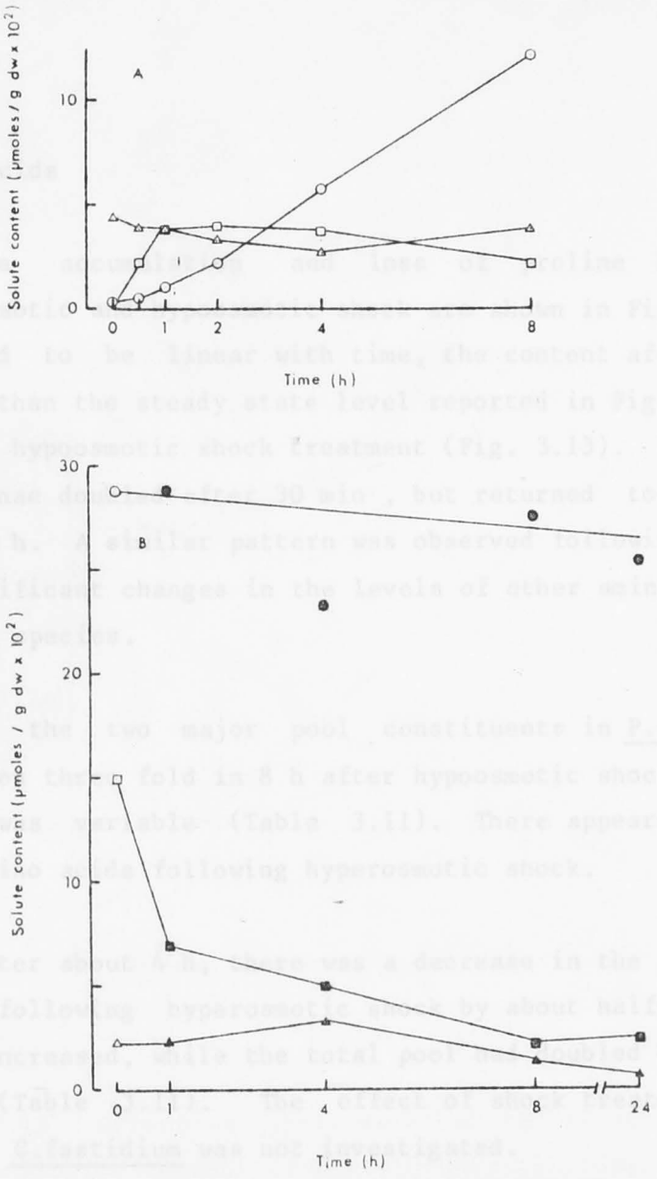


Fig. 3.12. Effect of hyperosmotic shock on the principal soluble carbohydrate of Penicillium chrysogenum (A) and of isoosmotic transfer to KCl on Chrysosporium fastidium (B). Glycerol (circles), glucose (squares) and mannitol (triangles).

Amino acids

The accumulation and loss of proline by P.cinnamomi after hyperosmotic and hypoosmotic shock are shown in Fig. 3.13. Accumulation appeared to be linear with time, the content after 8 h being somewhat higher than the steady state level reported in Fig. 3.6 or that at $time_o$ for the hypoosmotic shock treatment (Fig. 3.13). The alanine content of the hyphae doubled after 30 min , but returned to the original level after 2 h. A similar pattern was observed following hyperosmotic shock. No significant changes in the levels of other amino acids were apparent in this species.

Of the two major pool constituents in P.chrysogenum, aspartate increased three fold in 8 h after hypoosmotic shock, while the glutamate level was variable (Table 3.11). There appeared to be a decrease of both amino acids following hyperosmotic shock.

After about 4 h, there was a decrease in the total pool of amino acids following hyperosmotic shock by about half, but after 8 h it had again increased, while the total pool had doubled 8 h after hypoosmotic shock (Table 3.11). The effect of shock treatment on the amino acid pool in C.fastidium was not investigated.

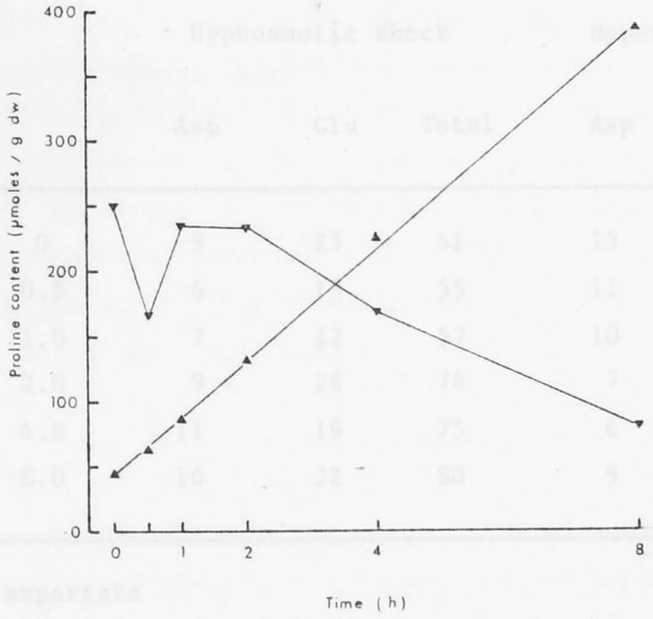


Fig. 3.13. Effect of hyperosmotic shock (▲) and hypoosmotic shock (▼) on the proline content of Phytophthora cinnamomi.

Table 3.11. The effect of hyperosmotic shock and hypoosmotic shock on the levels of the principal components and total amino acid pool in Penicillium chrysogenum.

Time (h)	Amino acid content (μmoles/g dw)					
	Hypoosmotic shock			Hyperosmotic shock		
	Asp	Glu	Total	Asp	Glu	Total
0	5	23	41	15	27	80
0.5	6	13	55	11	10	57
1.0	7	12	57	10	8	62
2.0	9	26	76	7	7	53
4.0	11	19	75	6	8	48
8.0	16	32	80	9	13	65

Asp = aspartate

Glu = glutamate

DISCUSSION

Osmotic shock experiments have been performed by a number of workers when investigating the mechanism of osmoregulation. This has generally led to a well defined sequence of events. Following hyperosmotic shock, water is lost with a subsequent decrease in volume in wall-less cells, and some degree of plasmolysis in walled cells. This is followed by a gradual return, usually to the initial volume or turgor, dependent on water influx as the concentration of osmotically active substances increases internally. If the cell behaves as a perfect osmometer, the volume change due to water loss can be described by the Boyle-Van't Hoff equation:

$$\psi_{\pi}(V - b) = \phi RTn_i$$

where ψ_{π} is the external potential, V the total cell volume, b the non-osmotic volume, ϕ the osmotic coefficient of the intracellular solute, n_i the number of molecules of that solute in the cell and R and T have their usual meanings. Thus this type of experiment may be used to determine non-osmotic volume and also, the degree of permeability of the external solute if a term is added for the reflection coefficient. In single cells this process can conveniently be followed by change in turbidity of cell suspensions or by electronic measurement of cell volume. In filamentous fungi the process must be inferred by measurements of osmotic potential accompanied by rates of change of internal osmotica.

Alemohammad and Knowles (1974) showed that the increase in turbidity in Escherichia coli was directly related to the osmotic potential of non-permeant solutes in the media, provided that allowance was made for the refractive index of the suspending solution. Rose (1975) did not make this correction and wrongly interpreted decreasing extinction as showing plasmolysis in osmophilic yeasts. The time interval for volume recovery was 15 to 180 min in the case of the alga Dunaliella parva (Gimmler et al., 1977). The process was independent of ATP which excludes the theory that it is dependent on glycerol

synthesis. It was concluded that small influxes of ions would probably account for this result. In another alga, Platymonas subcordiformis, there was a transient increase of ions to bridge the gap until mannitol was synthesised to balance the external potential (Kirst, 1977). Certainly the time course of the accumulation of glycerol in P.chrysogenum and C.fastidium was of the order of hours rather than minutes (Fig. 3.12). However there does not appear to have been a detectable influx of ions following hyperosmotic shock, so that equilibrium was not re-established for several hours. The organism must therefore be able to survive conditions of low turgor for such a period, presumably by the cessation of growth (Chapter 5). Brown (1978) considers that the rate, as well as the final yield, of osmoregulatory solutes are critical to the ability to respond to water stress. The more rapid volume recovery of the halotolerant yeast Debaryomyces hansenii than the non-tolerant yeast Saccharomyces cerevisiae following hyperosmotic shock was attributed to a better ability to produce osmotically active solutes (Nörkrans & Kylin, 1969).

In some species shock treatment has produced different results from steady state stress. Wyn Jones and Storey (1978) compared the relative importance of proline and glycine betaine under conditions of salt (NaCl) and water (PEG) stress in two cultivars of barley. A gradual incremental or pseudo steady state stress led to a greater accumulation of glycine betaine while hyperosmotic shock treatment resulted in higher levels of proline. Under steady state high salinity conditions, the proline level in the diatom Cyclotella cryptica was relatively low, and a rapid accumulation only occurred as a result of shock treatment (Liu & Hellebust, 1976a). Proline formation appeared to be a similar process in P.cinnamomi under both types of conditions (Fig. 3.6 & 3.13). Alanine, which in the present experiments also increased temporarily following both hyperosmotic and hypoosmotic shock, can be formed by transamination from other amino acids, thus it is likely to appear when proline turnover is high.

Protein appears to act as a sink for excess proline much as polysaccharide probably does for polyols. Following hypoosmotic shock to C.cryptica, there was an incorporation of ^{14}C from the accumulated proline into protein and also other cell constituents (Liu & Hellebust,

1976a). Leakage of proline probably did not occur since there was little discrepancy between the initial amount of label used and that finally accounted for in cell material. Decay of proline in P.cinnamomi was also a gradual process, unlikely to be associated with leakage (Fig. 3.13). Proline does however appear to leak from bacteria subjected to hypoosmotic shock (Gould & Measures, 1977) and the whole pool of amino acids from Escherichia coli (Britten & McClure, 1962). Leakage of mannitol from Dendryphiella salina (Jennings & Austin, 1973) but not Pyrenochaeta terrestris (Wright & Le Tourneau, 1965), and glycerol from Saccharomyces rouxii and S.cerevisiae (Brown, 1978) but not Dunaliella parva (Ben-Amotz & Avron, 1973), may be part of the osmoregulatory mechanism in these species. There was also a very rapid loss of K^+ from E.coli subjected to hypoosmotic shock (Epstein & Schultz, 1965).

The hypoosmotic shock experiments appear to have revealed a fundamental difference between C.fastidium and the other two species which may account for, if not explain, the obligate xerophily of the former. It is probable that such treatment induced irreversible changes in membrane permeability in C.fastidium resulting in loss of its cation selectivity and leakage of internal solutes. It is arguable whether death results from the loss of membrane integrity or is a cause of it. Tips of both species burst when subjected to this treatment (Chapter 5). An alternative explanation might be that healing is slower, or less effective, in C.fastidium so that more protoplasm is lost from the hyphae than can be tolerated. An increase in permeability and subsequent leakage of solutes at low water potentials was noted in conidia of Neurospora crassa, but the damage was not lethal (Charlang & Horowitz, 1974). They did not observe such changes in the more xerotolerant species Penicillium chrysogenum and Aspergillus nidulans.

Isoosmotic KCl did not appear to alter permeability and the cause of the inhibition remains unknown. The transitory appearance of erythritol in this experiment is of interest and coincides with the larger amounts of this compound found in the P.chrysogenum steady state KCl series than the glucose series (Fig. 3.5).

THE EFFECT OF POSITION IN THE COLONY ON THE HYPHAL SOLUTES OF *PENICILLIUM CHRYSOGENUM*

Glass Petri plates 14 cm in diameter contained 150 ml CYA, the potential of which was lowered with glucose to 0, -2 or -10 MPa. The plates with the medium were sterilised and then overlain with cellophane. Plates were inoculated centrally and colonies allowed to grow almost to the edge. Rings 2 cm wide were cut in the colony with three concentric 'biscuit cutters' soldered together, to give zones designated margin (M), -2 cm and -4 cm from the margin. Samples were taken from each of the three zones for ion, carbohydrate and amino acid analysis as well as hyphal osmotic potential and water content as described previously.

RESULTS

Osmotic potential

In these experiments there was no difference in the osmotic potentials of hyphae in the different parts of the colony (Table 3.11). This was in complete disagreement with the results reported in Chapter 2 and which were confirmed in subsequent experiments, where low turgor was found in older parts of the colony. The water content of the mycelium from 0 MPa increased with age but decreased in the -2 and -10 MPa treatments and a similar pattern of water content of whole colonies of different ages was seen also (data not shown). It is possible that in the larger (and older) colonies that the mat was no longer in contact with the agar and that some degree of drying had taken place. Alternatively, the surface area to volume ratio was greater in the smaller (9 cm) plates used in Chapter 2, so that nutrients may have been limiting in these but not in the large colonies.

Table 3.11. The effect of position in the colony on various parameters of the hyphae of Penicillium chrysogenum.

ψ_s (-MPa)	Zone	K^+	Na^+	Mg^{2+}	Ca^{2+}	Cl^-	$K^+:Na^+$	Fw:dw	ψ_{hypha} (-MPa)
		(μequiv/g dw)							
0	M	487	124	27	10	63	3.9	3.87	1.06
	-2	545	167	32	17	152	3.3	4.84	1.21
	-4	345	189	36	41	87	1.8	4.69	1.00
2	M	524	66	19	3	90	7.9	3.20	3.03
	-2	339	62	20	4	80	5.5	2.51	4.53
	-4	290	66	16	4	80	4.4	2.61	3.31
10	M	403	68	15	2	57	5.9	2.91	10.33
	-2	211	48	16	3	10	4.4	1.96	10.32
	-4	197	44	17	5	22	4.5	1.94	10.25

Ion content

The ion contents for the different treatments are shown also in Table 3.11. The K^+ content of the hyphae decreased with age. This was most noticeable in the -10 MPa glucose colonies where the hyphae near the centre contained half the K^+ present in the margin; this was the slowest growing of the three colonies and therefore the oldest in terms of time rather than position. The Na^+ content showed a different pattern in each of the three treatments; there was an increase with age in the 0 MPa colonies, the level remained approximately constant at -2 MPa and decreased in the -10 MPa treatment. However the $K^+:Na^+$ ratio, which appears to be a good indicator of hyphal growth as well as tolerance to low water potential, decreased with age in all three treatments.

The effect of age on the chloride content was not particularly

clear, although the values were appreciably lower in the older hyphae of the -10 MPa colonies. As with the steady state experiments, the Cl^- content was probably insufficient to balance the cations.

The Ca^{2+} content was again noticeably higher in the mycelium from the 0 MPa treatment compared to the colonies from -2 and -10 MPa. Mg^{2+} and Ca^{2+} remained relatively constant with age in the other two treatments.

Carbohydrate

The amounts of ethanol soluble carbohydrate present in the hyphae of different ages are presented in Table 3.12. Glucose and glycerol, which were the compounds proposed to respond to low water potential, were appreciably lower in the older hyphae than at the margin. This is in agreement with the phenomenon of hyphal growth which occurs predominantly at the tip (Chapter 4). The requirement for enzyme protection and high turgor will be greatest in the growth zone so that this result provides further evidence that these two compounds function as compatible solutes and osmoregulators.

Mannitol was highest at the margin in 0 and -2 MPa glucose colonies although not -10 MPa suggesting that its presence was associated with some process linked to growth, possibly respiration. Erythritol and probably arabitol were not correlated with age.

Amino acids

The total amino acid pool decreased with age in each of the three treatments (Table 3.12). The principal components, glutamate and aspartate, were also greatest at the margin (data not shown).

Table 3.12. The ethanol soluble carbohydrate content of hyphae from different positions in colonies of Penicillium chrysogenum grown at three different osmotic potentials.

ψ_s (-MPa)	Zone	Solute content (μmoles/g dw)						
		Gy	Gu	F	M	E	A	TAA
0	M	47	0	t	427	114	29	21
	-2	t	0	t	224	t	t	18
	-4	t	t	t	163	t	t	13
2	M	813	361	t	322	376	t	22
	-2	116	239	t	226	289	0	11
	-4	99	197	t	233	353	0	8
10	M	1859	786	t	103	87	t	5
	-2	646	467	0	135	71	13	8
	-4	647	530	0	152	126	23	6

t = trace
Gy = glycerol
Gu = glucose
F = fructose
M = mannitol
E = erythritol
A = arabitol
TAA = total amino acids

DISCUSSION

The phenomenon of apical growth means that the age of a cell can approximately be determined from its position in the colony. Thus the effects of age, which are more usually determined from experiments which are continuous in time, can be inferred from spatial distributions. The results of this experiment have confirmed the importance of glycerol and the K^+:Na^+ ratio for growth, the values of these being always greater at the margin than in the older parts of the colony. The osmoticum used in the medium, which in this case was glucose, was also present in greatest amounts at the margin.

Mannitol and erythritol were the principal higher polyols in the older parts of the colony, although there was less than at the margin except in the case of the -10 MPa glucose treatment. Glucose may have been limiting in the 0 MPa colonies but not at the lower potentials, so that there may have been some utilisation of polyols as respiratory substrates in the former.

Age has been found to affect polyol content of other species. In Dendryphiella salina mannitol was formed from glucose in both the lag and log phases following starvation, but arabitol did not appear until the onset of log phase (Holligan & Jennings, 1972a). In the storage and maintenance phases which follow exponential growth in liquid culture, the primary metabolites of D.salina, the two polyols, declined and secondary products, in this case malic acid, accumulated as the culture aged. Pyrenochaeta terrestris when grown in liquid culture on a number of different carbon sources was always found to contain the carbon source (except oligisaccharides) and mannitol during growth, but only mannitol remained in the non-growing cells (Wright & Le Tourneau, 1965). Other polyols may however have been lost in the washing procedure. A similar situation was observed in Debaryomyces hansenii where arabitol accumulation was greatest in the stationary phase cells grown in 2.7 molar NaCl (-14.6 MPa) while glycerol increased rapidly during logarithmic growth and virtually disappeared when the cells entered the stationary phase (Adler & Gustaffson, 1980). Decreasing activity of the

enzyme mannitol-1-phosphate dehydrogenase with age has been demonstrated in three species of fungi; Piricularia oryzae (Yamada et al., 1961), Pyrenochaeta terrestris (Aitken et al., 1969) and Sclerotinia sclerotiorum (Wang & Le Tourneau, 1972), which would presumably be associated with a decrease in mannitol content. Mannitol, and probably trehalose, decreased during germination and vegetative growth of Geotrichum candidum but accumulated again during arthrosporulation (da Costa & Niederpruem, 1980). Arabitol accumulated during germination and vegetative growth, but only when grown on 25% w/v glucose (ca -5 MPa) and not on 2% glucose.

The K^+:Na^+ ratios of all three treatments decreased with distance from the margin, largely as a result of the decrease in the amount of potassium, although there was also an increase in sodium in the 0 MPa treatment. This ratio in both Neurospora crassa and D.salina increased rapidly in the early stages of growth and regrowth (see Jennings, 1979); in shake cultures of D.salina the ratio started to decrease after 24 h (Galpin et al., 1978). In Escherichia coli also the K^+:Na^+ ratio increased during the early logarithmic phase and then decreased with age as a result both of decrease in K^+ content and increase in Na^+ (Schultz & Solomon, 1961). The ratio, in stationary phase cells, could be made to increase by resuspending them in fresh medium and the process was energy dependent.

Spatial distribution of ions along the first 200 μm of a single hypha has been examined by Galpin et al. (1978), using the technique of X-ray microanalysis. From work on D.salina, it appeared that the apex was not, as might have been expected, the most selective region and the K^+ content was greater and the Na^+ content lower some 50 μm behind the apex. The region behind this was also less selective. The K^+,Na^+ ATPase activity however was greatest in this older section some 200 μm behind the apex (Galpin & Jennings, 1975). High and constant concentrations of K^+ were found in the apex of the sporangiophore of Phycomyces blakesleeanus regardless of the water activity of the environment in the early stages of development, while older sporangiophores had a higher K^+ content at the base which was related to water activity (Cowan et al., 1972). ATPase was correlated with the region of high K^+ content in this species (Galpin & Jennings, 1976). Jennings (1979) has proposed that

ATP dependent K^+ pumps in the older parts of the hyphae might serve to generate a current flow along the hypha which could drive water and protoplasmic vesicles to the apex. Zalokar (1959) used cytochemical methods to demonstrate that tips (first 50 to 100 μm) of N.crassa were significantly richer in protein-bound sulphydryl groups and RNA than the rest of the hypha while glycogen was absent from the tips which would be consistent with the production of polyol osmoregulators and respiratory substrates in the growth zone.

Autolysis of old hyphae was observed in P.chrysogenum where the carbon source was limiting (Trinci & Righelato, 1970). This appeared to occur in some compartments of the hyphae so that regrowth was possible in others. They also observed intrahyphal hyphae growing in older parts of the colony, which if present in the experiments reported here, would alter the expected distribution of solutes within the colony. The changes in organic solutes in Aspergillus flavus during autolysis were followed by Lahoz et al. (1966). Mannitol, sugars and free amino acids all decreased rapidly in the hyphae whereas fat did not seem to be broken down.

THE EFFECT OF WATER POTENTIAL ON THE ACCUMULATION OF ORGANIC SOLUTES IN THREE LOWER FUNGI

A water mould tentatively identified as Aplanes sp., was isolated from Sullivan's Creek, Australian National University by baiting diluted creek water with millet seed. Mucor hiemalis and Pythium debaryanum were obtained from the Botany department, ANU. All three were maintained in culture on 1.7% cornmeal agar, and grown for this experiment in the usual way on CYA at 0 MPa, -0.5 MPa sucrose and -1.5 MPa sucrose. Hyphae were analysed for amino acids and ethanol soluble carbohydrate.

RESULTS

It can be seen from Table 3.13 that all three species accumulated proline as the water potential decreased. Amounts were lower than that found in Phytophthora cinnamomi (Fig. 3.6). The lowest potential, -1.5 MPa was very close to the growth limit of P.debaryanum, and colonies were extremely sparse. Thus the values from this treatment may not have been accurate. Glycine and alanine were also the other predominant components of the amino acid pool.

There was some difference between the species in the composition of the carbohydrate pool. M.hiemalis and Aplanes sp. contained only sucrose, whereas in P.debaryanum sucrose had obviously been hydrolysed to the constituent monosaccharides. Some mannitol was also detected in this species, but none of the three appeared to contain glycerol. The pentitol arabitol was present only in P.debaryanum, together with a trace of erythritol.

Table 3.13. The effect of water potential on the organic solutes of three lower fungi.

Species	ψ_s (-MPa)	Solute content (μmole/g dw)							
		Pro	Gly	Ala	Ara	Fru	Glu	Man	Suc
M.hiemalis	0	15.1	8.0	32.7	0	0	t	0	t
	0.5	59.3	11.4	78.9	0	0	t	0	80.1
	1.5	91.0	9.3	77.2	0	0	t	0	333.1
P.debaryanum	0	15.3	4.9	11.7	0	t	t	0	0
	0.5	186.6	10.3	30.8	t	193.0	280.0	t	53.9
	1.5	56.2	3.4	20.3	26.4	790.4	912.6	135.0	58.9
Aplanes sp.	0	23.1	6.7	19.0	0	0	0	0	0
	0.5	67.5	7.1	23.0	0	t	t	t	160.9
	1.5	156.3	4.2	19.8	0	t	48.9	t	410.5

Pro = proline
Gly = glycine
Ala = alanine
Ara = arabitol
Fru = fructose
Glu = glucose
Man = mannitol
Suc = sucrose

DISCUSSION

The two principal, but very distinct, classes of the lower fungi are the Mastigomycotina and the Zygomycotina. The former are water moulds with coenocytic mycelium and biflagellate zoospores while the latter are terrestrial fungi and do not produce motile spores. The genus Aplanes of the Saprolegniaceae are ubiquitous aquatic fungi and the majority are saprophytic and of little economic importance. The Pythiaceae may be aquatic or terrestrial. P.debaryanum used in this experiment is an important plant parasite. The genus Pythium differs from that of Phytophthora in the same family, in its method of sporangial germination. The class Zygomycetes contain the family Mucoraceae to which the genus Mucor belongs. These are the well known saprophytic pin moulds. M.hiemalis was used in this experiment. Like P.cinnamomi, all three species are coenocytic.

Glycerol was not detected in this experiment but twenty-three varieties of Mucor have been shown to produce large amounts of glycerol which was increased by the addition of NaHSO or Na_2CO_3; the optimum concentrations were 6% and 4% respectively (ca -4.0 and -2.9 MPa (Takahashi & Asai, 1933). A number of Mucor species are known to accumulate mannitol, although M.hiemalis was not among the test species (Boonsaeng et al., 1976). However the activity of mannitol-1-phosphate dehydrogenase in M.rouxii was much lower than in the Ascomycetes. The absence of a pentitol in M.hiemalis agrees with the results of Curtis et al. (1980) who found ribitol to be present in a number of Mucorales but not in this species.

M.hiemalis was the most xerotolerant of the three species tested here, and this was consistent with the survey of Tresner and Hayes (1971) on the NaCl tolerance of fungi. Saprolegniaceae are probably the least tolerant, and they were not found in estuaries with a salinity of greater than 2.8% (-0.01 MPa) although higher salinities were tolerated in culture (TeStrake, 1959).

It is particulary interesting that the amino acid hydroxyproline

was recently thought to occur only as a constituent of the cell wall protein of Oomycetes (Burnett, 1976). This may have been simply an artifact of analysis since there is now a report of its occurrence in a Basidiomycete (Cameron & Taylor, 1976). In animal tissues, hydroxyproline occurs principally in collagen. Its biosynthesis is not fully understood. Free proline does not appear to be the substrate for the hydroxylation reaction, and the sequence is probably:

proline → protocollagen → hydroxylated protocollagen → collagen

There is some suggestion that the presence of hydroxyproline might indicate an 'elastin' like protein in the cell walls which would be important in organising wall growth by intersusception between microfibrils (Burnett, 1976). A link between the accumulation of proline under water stress and some adaptation of wall structure to allow growth to continue under such conditions is of course purely speculative.

That lower fungi should differ from other fungi in their mode of osmoregulation is not surprising. Their dependence on an aquatic reproductive form suggests that their evolutionary origins are different from other fungi, and they are also known to differ physiologically. The different polysaccharides employed as constituents of the cell wall is one example and the organisation of the hyphal apex is another (Burnett, 1976). Cantino (1966) has accounted for the physiological evolution of saprophytic water moulds in terms of successive loss of synthetic capacity.

GENERAL DISCUSSION

The greater tolerance of Saccharomyces rouxii than S.cerevisiae to low water potential has been attributed to the ability of the former to retain glycerol more efficiently, rather than to greater production (Brown, 1978). Although leakage of glycerol from P.chrysogenum and C.fastidium has not been investigated, the greater ability of the latter to increase its K^+:Na^+ ratio as the water potential fell (Fig. 3.2) points to a difference between the membrane characteristics of the two species. Glycerol does not appear to leak from Dunaliella parva (Ben-Amotz & Avron, 1973) and regulation was thought to be accomplished by metabolic conversion, as has been suggested here for P.chrysogenum and C.fastidium. However the problem of how glycerol is retained within the cell against large concentration gradients remains unsolved.

Membranes are undoubtedly highly permeable to glycerol, a feature which is partly associated with the small size of this molecule (Wright & Diamond, 1969). Gancedo et al. (1968) have shown that Candida utilis was more permeable to glycerol than S.cerevisiae, and Corry (1976b) has demonstrated that two osmophilic yeasts were more permeable to glycerol than to glucose, fructose, sucrose or sorbitol. The proportion of the cell penetrated by glycerol was close to that penetrated by water in Escherichia coli, but the rate of glycerol penetration was slower than that of water, and was probably by simple diffusion or, in induced cells, by facilitated diffusion (Alemohammad & Knowles, 1974). Brown (1978) suggests that glycerol uptake is probably an active process in S.rouxii but not in S.cerevisiae. There was an energy linked component of uptake of a similar molecule ethylene glycol in both Debaryomyces hansenii and S.cerevisiae (Adler & Liljenberg, 1980). Proline also is readily exchangeable across bacterial membranes (Gould & Measures, 1977); they argued that accumulation was therefore unlikely to be achieved by a decrease in permeability.

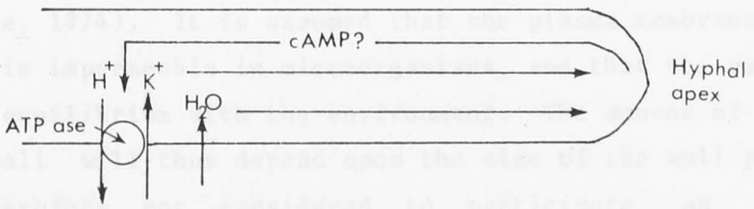
Some attempts have been made to correlate wall structure and membrane characteristics with osmotic adaptation. Trevithick and Metzenberg (1966) have shown that the wall was thinner and the pores

abnormally large in an osmotic mutant of Neurospora crassa. The walls of a number of these mutants of N.crassa which were unable to grow at low water potentials were also analysed by Livingston (1969) and she found differences in composition of polysaccharide in all the mutant strains as compared with the wild type. Adler and Liljenberg (1980) found that D.hansenii had a higher ratio of free sterols to phospholipds than S.cerevisiae, and that an increase in salinity had little effect on the fatty acid composition of the former species whereas there were significant changes in S.cerevisiae. Similarly, Koh (1975) has analysed the cell envelopes of S.rouxii and of a mutant strain which was unable to grow at high water potentials. He noted differences in the ratios of glucose to mannose and also in the hexosamine content between the two. There was also a greater proportion of unsaturated fatty acids in the lipid fraction of the osmophilic mutant. It was suggested that greater membrane fluidity might result from this difference and account for the tendency to lyse in response to hypoosmotic shock, as well as other morphological differences. Another example of an effect of low water potential on membrane characteristics was the alteration, by high KCl concentrations, of permeability of Saccharomyces spp. to phenol red and potassium ferricyanide, both of which are normally excluded (Lillehoj & Ottolengi, 1967). Such results, however are only suggestive, and no clear relationship between membrane function and tolerance of water potential has yet been established.

Although some organic molecules may permeate readily, fungi generally have a low passive permeability to ions (Slayman, 1970). Uptake will therefore generally be an active, energy dependent process. Slayman and co-workers using N.crassa have elucidated a mechanism of active uptake of solutes. They have demonstrated that there is an electrogenic pump which is driven by ATP and generates an efflux of protons. This flux serves to stabilise the internal pH and, more importantly, to set up a potential difference of about -200 mV across the membrane. This potential then serves as the main energy distributor for transport (Slayman & Gradman, 1975), and drives the uptake of different substances coupled with H^+ influx. Glucose uptake has been directly demonstrated to occur by this mechanism and there is indirect evidence for such uptake of amino acids, phosphate and potassium, and most recently monosaccharides and polyols in a yeast (Hofer & Misra,

1978). The system differs from that of animal cells in that H^+ and not Na^+ is the extruded cation, and that membrane potential and not ionic concentration gradient, drives the transport system (Slayman, 1970). However there may be some Na^+ extrusion linked with K^+ uptake, particularly in marine organisms e.g. yeasts (Norkrans & Kylin, 1969) and Dendryphiella salina (Jennings, 1979).

Change in turgor may itself be the trigger for uptake or synthesis; Racusen et al. (1977) found that electrical properties of plant protoplasts could be altered by osmotic shock. Twenty five per cent of the ATP turnover in N.crassa is required to drive the proton pumping system (Slayman et al., 1973), but while ATP drives the pump there is some evidence that the regulatory agent is cyclic (3,5-) adenosine monophosphate (cAMP). cAMP is already known to be involved in the control of growth morphology (Scott & Solomon, 1975). Jennings (1979) has speculated on how the various factors of hyphal growth, potassium transport and ATP:AMP might be interrelated. His thoughts are best summarised by his own diagram:



The contribution of both ions and organic solutes to the osmotic potential of most plant species is now generally accepted. For instance the careful work of Gimmler and Schirling (1978) has demonstrated that Na^+ taken up from the saline medium as well as glycerol synthesised by Dunaliella both contribute significantly. This has also been demonstrated to be the case in the fungal species of this study. An attempt has been made to sum the contribution of the various solutes to determine whether the external osmotic potential has been accounted for (Table 3.14). Howevever, such estimates of actual concentrations require a number of assumptions and must be applied with caution.

Firstly calculation of hyphal concentrations requires that the volume of the cell occupied by osmotically active solutes be known. In

liquid cultures of microorganisms, this is usually determined from concentrations of supposedly impermeable substances such as inulin, phosphate or sorbitol, and in fungi it is generally equated with the proportion of the cell external to the plasma membrane and so is equivalent to the cell wall (Burnett, 1976). The inulin impermeable space of N.crassa grown in liquid culture of high water potential has been determined as 16.7% (Slayman & Tatum, 1964), although this would have included interstitial as well as periplasmic space; electron micrographs showed the cell wall to occupy approximately 20% of the cell volume. This value has also been used to determine hyphal concentrations in D.salina grown under similar conditions (e.g. McDermott & Jennings, 1976).

Only in bacteria has the proportion of the wall or periplasmic space been estimated by measuring the difference between the concentration of a large molecule inaccessible to the wall pores (dextran) and a molecule (phosphate, stachyose, sucrose) impermeable to the plasma membrane. The values obtained were 8% in Staphylococcus aureus (Mitchell & Moyle, 1956) and 32% in E.coli (Alemohammad & Knowles, 1974). It is assumed that the plasma membrane is the barrier which is impermeable in microorganisms, and that the wall will therefore be in equilibrium with the environment. The amount of water retained in the wall will thus depend upon the size of the wall pores. Wall water is therefore not considered to participate as a solvent for intracellular solutes, although it has sometimes been argued that the fixed charges of the walls may be balanced by some of the cellular cations. Galpin et al. (1978) however did not consider this to be a large proportion in D.salina.

Measurements of inulin impermeable space have not been attempted in these experiments. However it is possible that different culture conditions (solid vs liquid) or low water potential per se might affect wall thickness or wall:cell ratios. Experiments to test this possibility were carried out using electronmicrographs of thin sections and are reported in Chapter 5. The results have been used to calculate a correction factor for non-solvent water in the walls.

In addition to wall water, it appears that a certain proportion of

the water within the cytoplasm itself which is unavailable as a solvent for osmotically active substances (Cooke & Kuntz, 1974). This proportion, which may be of the order of 20 to 30%, is thought to form a monolayer on the surface of macromolecules such that its properties are altered and it is unable to act as solvent. Whether this non-solvent water corresponds to the old concept of bound water is not known. The latter was defined as the water removed by heat but not by drying in vacuo at room temperature. Whatever the physiological interpretation, this bound water was found to increase in the mycelium of Aspergillus niger directly with the concentration of the solution on which it was grown (Todd & Levitt, 1951). The temperature of drying also affected the water lost, and bound water constituted a greater proportion when the mycelium was dried at 110°C than when it was dried at 80°C. In all the experiments of the present study, the dry weights were obtained at 85°C, a temperature generally adopted for fungal material (Cochrane, 1958). A more accurate measure of the total water content might therefore have been obtained by drying at a higher temperature. A value of 25% non-solvent water has been assumed to calculate the concentrations shown in Table 3.14.

The estimates of concentrations did not generally add up to the measured osmotic potential of the hyphae, particularly those grown on glucose osmotica (Table 3.14). This may be due partly to errors in measurements of glycerol and proline where internal standards were not used, and consequently amounts may have been underestimated. The assumption of 25% non-solvent water in the cytoplasm may also vary both between species and at different potentials. Finally, some osmotically active substances, such as free organic acids, may not have been detected by these analyses.

Table 3.14. Estimated concentrations of some hyphal solutes in P.cinnamomi, P.chrysogenum and C.fastidium.

Solute	Measured content (μmoles/g dw or μequiv/g/dw)	Estimated concentration (molal)*	ϕ**	ψ_{hypha} (-MPa)
A. P.cinnamomi -2 MPa sucrose				
Na^+	107	0.131	0.93	0.605
K^+	220	0.269	0.91	1.214
proline	329	0.402	1.0	0.997
sucrose	344	0.420	1.0	1.042
			calculated	3.858
			observed	3.15

* Calculated from fw:dw 2.78, wall water 29% and non-solvent cytoplasmic water 25%.

B. P.cinnamomi -2 MPa KCl				
Na^+	80	0.108	0.93	0.498
K^+	999	1.349	0.90	6.022
proline	263	0.355	1.0	0.880
			calculated	7.40
			observed	3.43

* Calculated from fw:dw 2.61, wall water 29% and non-solvent cytoplasmic water 25%.

Table 3.14 cont'd.

C. P.chrysogenum -10 MPa glucose

Na^+	65	0.065	0.93	3.0
K^+	389	0.387	0.90	1.728
glycerol	1279 (2291)	1.273 (2.28)	1.02(1.03)	3.157(5.65)
glucose	702	0.699		1.720
mannitol	243	0.242		0.627
erythritol	118	0.117	1.0	0.290
			calculated	7.822 (10.32)
			observed	13.36

* Calculated from fw:dw 2.50, wall water 8% and non-solvent cytoplasmic water 25%.

D. P.chrysogenum -10 MPa KCl

Na^+	43	0.040	0.93	0.185
K^+	2210	2.049	0.91	9.248
glycerol	780	0.723	1.01	1.793
mannitol	169	0.157		0.423
erythritol	134	0.124	1.0	0.308
				11.957
				12.74

* Calculated from fw:dw 2.61, wall water 8% and non-solvent cytoplasmic water 25%.

Table 3.14 cont'd.

E. C.fastidium -20 MPa glucose				
Na^+	23	0.020	0.93	0.092
K^+	345	0.299	0.91	1.350
glycerol	2250	1.952	1.02	4.851
fructose	1340	1.163		2.880
glucose	1427	1.238		3.080
mannitol	147	0.128		0.347
arabitol	80	0.069	1.0	0.171
			calculated	12.761
			observed	23.89

* Calculated from fw:dw 2.72, wall water 8% and non-solvent cytoplasmic water 25%.

** Na and K as chlorides, and sucrose from Robinson and Stokes (1955); glucose and fructose from Norrish (1966) as described in Chapter 2; mannitol by psychrometry (Appendix 2); glycerol from Scatchard, et al. (1938); proline, arabitol and erythritol assumed equal to 1.0.

In other microorganisms, on some occasions, the identified osmoregulatory solutes have been judged to account for the external osmotic potential. Proline in Cyclotella cryptica (Liu & Hellebust, 1976a) or proline and sorbitol in Stichococcus bacillaris (Brown & Hellebust, 1978) did not account for the external osmolarity, although in both cases the concentrations were probably underestimated since water content was calculated on the basis of whole cell volume and also other solutes were not taken into account. Dehydration and proline accumulation were thought to account for the presence of 1.8 molar NaCl (-9.0 MPa) in the medium in Staphylococcus aureus (Koujima et al., 1978), but proline and glutamate accounted for only 25% of the total solutes of Vibrio alginolyticus at 1.5 molar NaCl (-7.3 MPa); the glycerol concentration was insignificant (Unemoto & Hayashi, 1979). Proline and sucrose did not account for the osmoregulation of the green alga Chlorella emersonii at high NaCl concentrations (Setter & Greenway, 1979). In D.hansenii, the solutes measured (Na^+, K^+, arabitol, glycerol and total amino acids) were approximately equivalent to the potential of the medium (2.7 molar NaCl, -14.6 MPa) (Adler, 1979).

4. THE EFFECT OF WATER POTENTIAL ON RESPIRATION AND THE ACTIVITY OF A RESPIRATORY ENZYME

INTRODUCTION

Filamentous fungi are almost entirely aerobic organisms. Oxygen is therefore principally required as the terminal electron acceptor in the respiratory chain. Provided that phosphorylation associated with electron transfer is not uncoupled, respiration is a measure of the energy requirement of the organism. This also assumes that respiration is an efficient process and does not generate ATP which is wasted. Accumulation, whether of an external solute or an internally synthesised metabolite, will require energy in addition to the usual anabolic requirement for growth. It is therefore pertinent to examine the relationships between external water potential, respiration and growth.

Respiration of microorganisms has conventionally been measured manometrically in a Warburg apparatus for which liquid culture is necessary. An electrolytic respirometer has been used in these experiments because of its suitability for work with whole colonies such as have been employed throughout this study. The characteristic radial growth of filamentous fungi on solid media allows respiration attributed to growth at the margin, and maintenance in the remainder of the colony, to be distinguished. Both steady state and shock experiments of the type described in the previous chapter have been carried out.

The similarity between the water relations of growth and the water relations of respiration observed here and by other workers, suggests that respiration may be the factor which limits the rate of growth at low water potential. The term respiration as used here includes the entire oxidative process. To determine whether water potential affects the metabolic machinery i.e. the enzymes in the intermediate steps of oxidation, or only the final demand for oxygen, the effect of water

potential on a respiratory enzyme of the tricarboxylic acid cycle, nicotinamide-adenine dinucleotide phosphate (NADP) specific isocitrate dehydrogenase, has been examined. This enzyme was chosen because it is easily assayed and becasue it has been investigated in this respect in other organisms. Also it does not use as substrate any of the solutes found to accumulate in hyphae in response to low water potential.

In vitro enzyme systems are inhibited by higher potentials (or salt concentrations) than those which limit growth in many species. Four possible explanations have been put forward:

1. In vitro assay conditions or damage during extraction mean that the system does not resemble in vivo conditions (Greenway & Osmond, 1972).

2. Solute distribution within the cell is not even, so that enzymes may be localized in regions of low inhibitor content (Greenway & Osmond, 1972).

3. Enzyme inhibition is substrate dependent, so that if substrate level in vivo is increased in response to high salinity, the inhibition is overcome (Greenway & Sims, 1974).

4. Salt is excluded from the cell (Borowitzka & Brown, 1974).

The first possibility can never be completely discounted. The second possibility can be examined in fungi, because there is no equivalent, at least in the growing hyphal tip, to the large vacuole of higher plant cells, so that compartmentalisation is unlikely. The third possibility was considered by ensuring that a non-saturating substrate concentration was used in the assays. Finally it has been demonstrated in the previous chapter that salt is not excluded from hyphae.

It has frequently been shown that the conditions of growth do not alter the characteristics of the extracted enzymes (e.g. Johnson et al., 1968; Greenway & Osmond, 1972; Liu & Hellebust, 1976b; Adler,

1978). This was tested with extracts of P.cinnamomi grown at several water potentials. Then the effects of some of the solutes identified in the previous chapter on the activity of isocitrate dehydrogenase extracted from each of the three species were examined.

MATERIALS AND METHODS

Fungi and media

Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium were grown as previously described. Media were also prepared in the same way except that all plates were poured with an electric pump (Medos Univol 1000) to ensure that volumes were as similar as possible.

Respiration

Respiration of whole colonies was measured as oxygen uptake by an electrolytic respirometer. The apparatus was based on the design of Birch and Melville (1969) and part of it is illustrated in Plate 4.1.

Briefly, oxygen used by fungal colonies in a sealed respirometer chamber caused the level of an electrolyte to rise. When contact was made with an electrode, an electrolytic current was switched on. The duration of the current was recorded on an electric clock until the oxygen replaced by electrolysis caused the level of the electrolyte to fall below the switching electrode. Respiration was calculated from the duration and rate of electrolysis. Electrolytic currents in the range 15 to 90 mA were used in order to produce about 20 min electrolysis per hour. If respiration rates were too high, the number of colonies in the chamber was also reduced. Stable voltage was supplied to each unit from individual 12 V transformers and the AC current to each was rectified with a diode bridge.

The respiration chambers were made from Perspex cylinders 10 cm in diameter and 11 cm high. Five 9 cm diameter petri plates, separated by Perspex supports, were stacked in each chamber. At the bottom of each stack was a petri dish containing 40 ml 2.5 N NaOH to remove carbon dioxide. The amount of alkali remaining was checked with an indicator. Each respirometer was connected via a glass bell to the electrolyte and electrolytic and switching electrodes. The bell served to separate the hydrogen and oxygen evolved so that only oxygen was returned to the chamber.

Each chamber was also connected, via a three way tap, to a pressure compensating device. This part of the apparatus was designed to control fluctuations in the level of the electrolyte due to changes in the atmospheric pressure and temperature. A control respirometer chamber was set up, containing five uninoculated plates and alkali, of identical volume to the experimental chambers. In this case there were two switching electrodes, one placed 2 mm above the other. When both electrodes were immersed, a reversible motor geared to rotate a shaft attached to a series of pistons and cylinders connected to the experimental chambers, was switched on so that the pistons were depressed and the levels of the electrolyte in all the chambers was moved down. When only one of the pair of electrodes in the control chamber made contact, the motor was switched off. Similarily, when a decrease in pressure caused the electrolyte level to fall below both electrodes, the motor operated in the reverse direction, and the pistons and hence the electrolyte were raised. Two pressure compensators were used; one was connected to three of the experimental chambers, the other to two. The whole apparatus was kept in a constant temperature room at 25°C ± 0.5°C to provide uniform conditions for fungal growth.

The instrument was originally designed for continuous recording, and was used as such by Wilson and Griffin (1975a; 1975b) to measure respiration by soil microorganisms. They recorded growth from control colonies not in chambers. There were a number of drawbacks associated with continuous recording:

1. Respiration rates could not be related to the size or weight of the colony respiring. Growth rates of

P.cinnamomi particularly, were very variable within treatments so that controls were not reliable indicators of actual biomass in the chambers.

2. The apparatus was set up in only semi-sterile conditions so that there was a risk of contamination during long experiments.

3. Alkali was used up during the experiment; continuous recording meant that the alkali had to be changed with the ensuing risk of contamination.

4. It is possible that removal of carbon dioxide from the atmosphere in the chamber and the resulting alteration of gas partial pressures may affect growth. There was some indication in preliminary experiments that growth rate and colony morphology were altered by prolonged incubation in the chamber. Griffin (1972) has reviwed the evidence concerning the effect of atmospheric conditions on fungi.

5. There was a risk of leaks and mechanical or electrical failures developing during the course of a long experiment.

6. It is possible that there may have been an alteration of the water potential of the media by isopeistic equilibration with the alkali; 2.5 N NaOH had an osmotic potential of -13 MPa.

7. Inconvenience of recording over a 24 h period.

For these reasons, the apparatus was operated in a discontinuous mode, so that respiration rate and not cumulative respiration was measured.

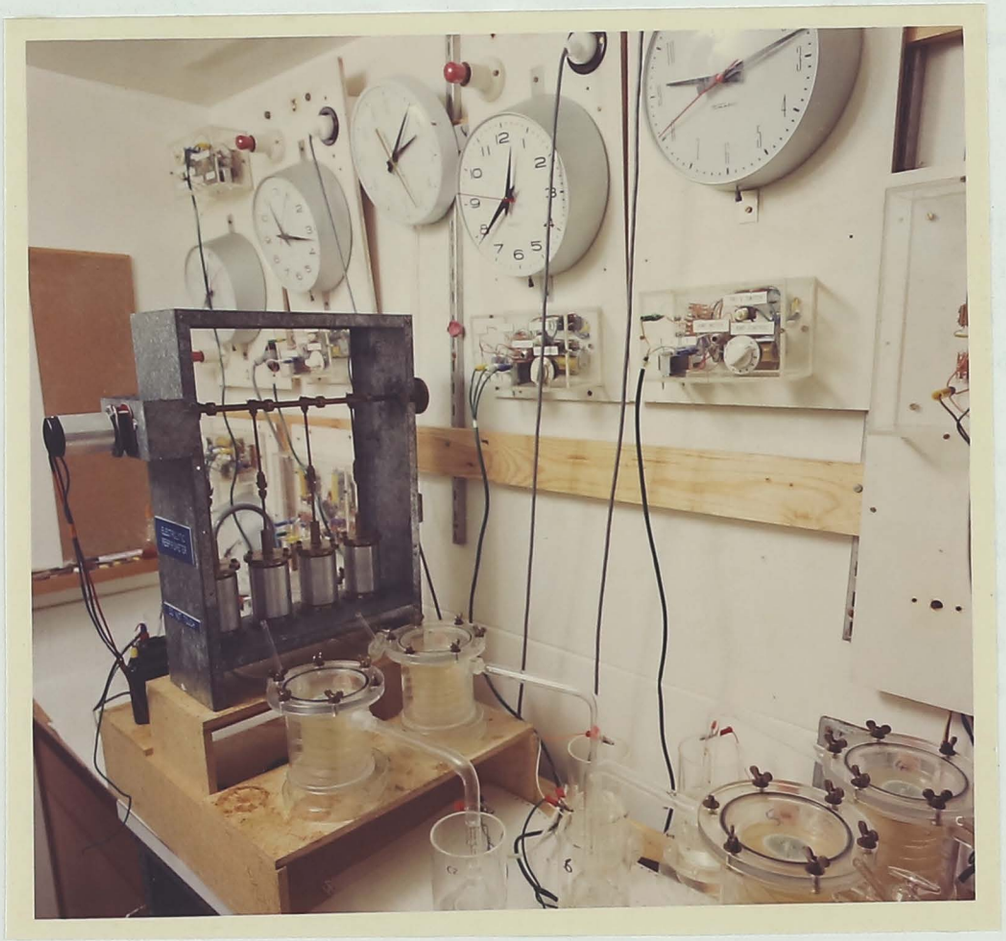


Plate 4.1. Electrolytic respirometer and pressure compensator.

Enzyme extraction

Prior to extraction, colonies were removed from the cellophane, blotted gently and weighed. If necessary the mats were coarsely chopped with a scalpel, then placed in the chilled 50 ml cell of a Sorvall Omni-Mixer homogeniser containing 3 ml ice cold buffer per gm colony weight, and homogenised for 30 sec on ice. The homogenate was transferred to the chilled 40 ml cell of a French press and the hyphae were ruptured at 103 MPa (15,000 lbs/sq in). The resulting suspension was centrifuged for 45 min at 27000 g to sediment unbroken fragments and wall debris. A clear, pale coloured supernatant was obtained which was used as a crude extract of isocitrate dehydrogenase.

Johnson et al. (1968) found that activity of glucose-6-phosphate dehydogenase activity from Dunaliella viridis was completely inhibited by extraction in buffer adjusted to the same salt concentration as the growth medium. An experiment was therefore conducted to determine whether the potential of the extraction buffer was critical. Extracts from P.chrysogenum grown under three different conditions were extracted in buffer containing several different osmotica. All the extracts were assayed at -0.5 MPa. The results are shown in Table 4.1. Although the potential of the extraction buffer or the osmoticum type did not appear to alter the activity of the enzyme from the high water potential, activity from material grown at -2.5 MPa was greatest when extracted in buffer at the same potential as the growth medium and with the same osmoticum. All buffers for extraction were therefore adjusted accordingly.

Table 4.1. The effect of the osmotic potential of the extraction buffer on the isocitrate dehydrogenase activity of Penicillium chrysogenum.

ψ_s(growth) (-MPa)	ψ_s(extraction) (-MPa)	Relative activity (specific)
0	0	100
0	1 (glycerol)	100
0	2.5 (KCl)	100
2.5 (KCl)	0	100
2.5 (KCl)	1 (glycerol)	131
2.5 (KCl)	2.5 (KCl)	154
2.5 (glucose)	2.5 (KCl)	62
2.5 (glucose)	2.5 (glucose)	67

The pH for optimum isocitrate dehydrogenase activity from all three species lay between pH 8.5 and 9.0 (data not shown). All extractions and assays were therefore performed at pH 8.6. N,N-bis (2-hydroxyethyl)glycine buffer (bicine) was used and adjusted to the correct pH with KOH after the potential had been adjusted.

The stability of the enzyme was dependent on the presence of magnesium in the extraction buffer. It was necessary to add 5 millimolar $MgCl_2$ to the extraction buffer for P.cinnamomi and 20 millimolar for the other two species.

Enzyme assay

NADP-specific isocitrate dehydrogenase activity (EC 1.1.1.42) was assayed according to the procedure of Reeves et al. (1971) with some modifications. Final concentrations of reagents in the 3 ml cuvette were:

50 millimolar	Bicine buffer, pH 8.6
2 millimolar	$MnCl_2$
0.5 millimolar	NADP
0.5 millimolar	DL isocitrate
enzyme	(1 to 3 mg protein)

There was no difference in activity if 2 millimolar $MnCl_2$ was replaced by 10 millimolar $MgCl_2$. The enzyme was kept on ice during the experiment but all the other reagents were at 25°C. The reaction was started by the addition of NADP, and the reduction followed at 340 nm with a Pye Unicam SP 1800 spectrophotometer fitted with a water jacketed compartment which was kept at 25°C. The velocity was calculated from the change in absorbance during the first minute. Wherever possible, results were the means of two, or if necessary, more assays. The enzyme activity was assayed at different osmotic potentials by adding the appropriate solute to the assay buffer. After the assay, each reaction mixture was retained and the actual osmotic potential of the solution measured by psychrometry as described in Chapter 2. The protein content of the enzyme extract was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Enzyme activity was assayed at different isocitrate concentrations and it was confirmed that 0.5 millimolar was non-saturating for each of the three species (data not shown).

THE EFFECT OF OSMOTIC STRESS ON STEADY STATE RESPIRATION

Phytophthora cinnamomi was grown on sucrose or KCl plates as before, at potentials as low as -2 MPa; Penicillium chrysogenum on glucose or KCl to -10 MPa and Chrysosporium fastidium on glucose only, to -20 MPa. Plates were incubated at 25°C until the lag phase had passed and sufficient biomass from which to record respiration had developed; 2 days in the case of P.cinnamomi, 3 days for P.chrysogenum and 6 days for C.fastidium. Respiration rates were then measured daily in the case of P.cinnamomi, every 2 days for P.chrysogenum and every 3 days for C.fastidium.

Respiration rates were measured by placing five colonies of each potential into a respiration chamber, with five uninoculated plates from the same batch in the control chamber. The chambers were sealed and allowed to equilibrate for 2 hours, and the electrolytic current adjusted to give a suitable duration of electrolysis. Respiration was recorded over a period of 4 or 5 hours. The plates were then removed from the chamber, and the radius, wet and dry weight of each colony determined in the usual way, so that both whole colony and specific respiration rates could be obtained. Unless mentioned otherwise, results were from single experiments, each value being a mean obtained from the five (or less) colonies contained in each respiration chamber.

RESULTS

Lowered water potential decreased the respiration rate of whole colonies ($Q\mu lO_2$/colony/h) of the same age (Figs. 4.1 & 4.2). This is in agreement with the results obtained by Wilson & Griffin (1975b) for several other species of fungi, and parallels the reduction in growth rate with decreasing water potential (Chapter 2). However, if the

respiration rate of colonies of the same radius (21 ± 2 mm) but hence of different ages, are compared, a similar pattern exists (Figs. 4.1 & 4.2). The curve resembles the growth curve; that is a maximum rate was observed at a potential below the highest tested, except possibly in P.cinnamomi. Thus it seems that there was a correlation between the respiration rate of a colony of a given radius (or weight) and the growth rate of that colony. Both radial growth rate and increase in dry weight on a daily basis have also been plotted in Figs. 4.1 and 4.2, and the two curves are similar. The linear correlation coefficients between respiration and growth rates and their significance are given in Table 4.2. The poor level of correlation in P.cinnamomi was probably due to the apparent inhibition of growth which occurred in this experiment at -0.5 MPa sucrose, instead of the stimulation noted previously (Fig. 2.2).

Table 4.2. The linear correlation coefficients between colony respiration rate (colonies of equal radius) and two measures of growth rate.

Species	dQ/dt§ vs dW/dt†	dQ/dt vs dR/dt††
P.cinnamomi	0.49	0.43
P.chrysogenum	0.85*	0.92*
C.fastidium	0.96*	0.90*

* Significant (P = 0.05).

§ µl O_2/colony/h

† mg dw/24 h

†† mm/24 h

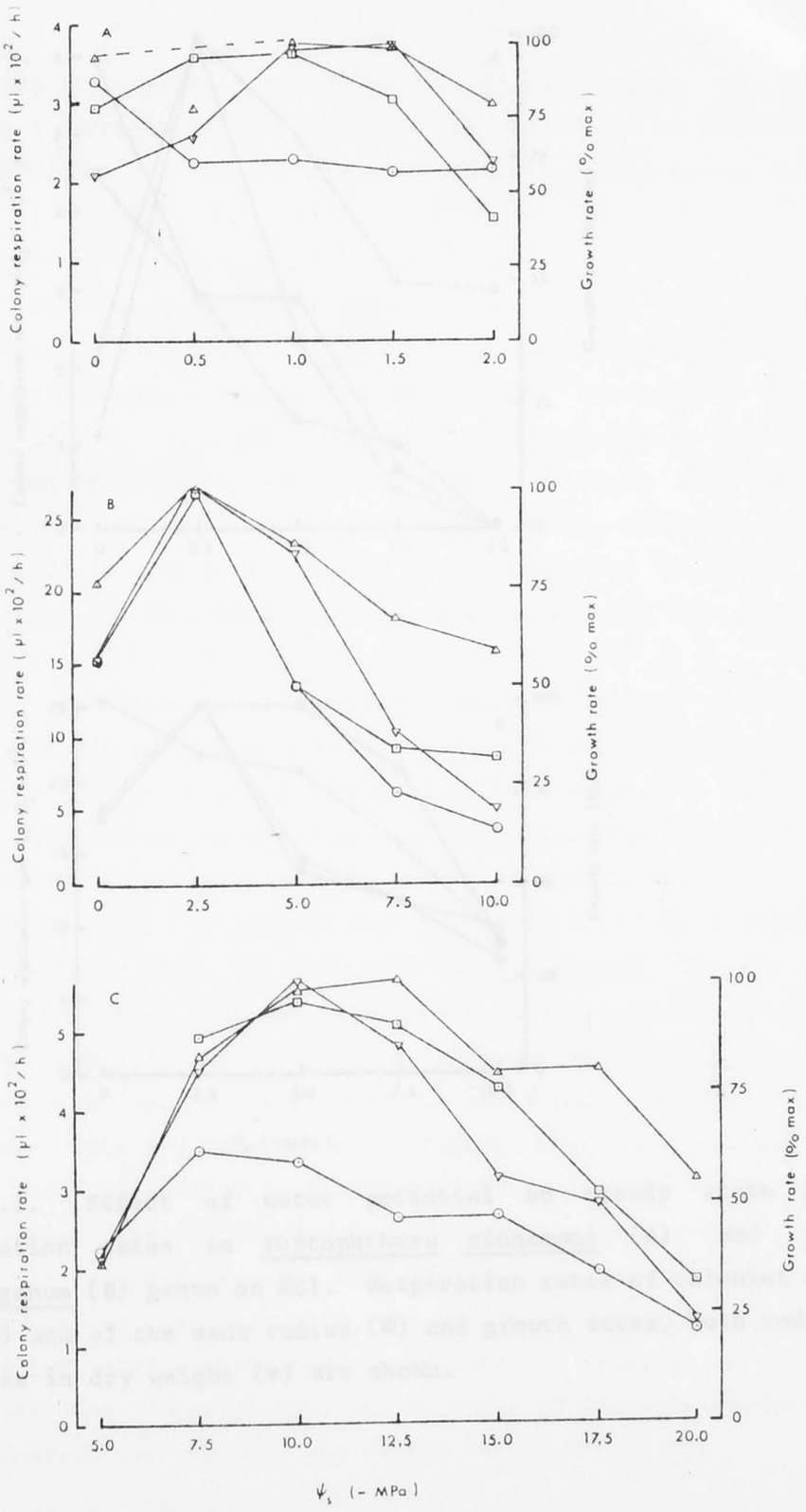


Fig. 4.1. Effect of water potential on steady state growth and respiration rates in Phytophthora cinnamomi (A), Penicillium chrysogenum (B) and Chrysosporium fastidium (C) grown on sugar. Respiration rates of colonies of the same age (○) and of the same size (□) and growth rates, both radial (△) and increase in dry weight (▽) are shown.

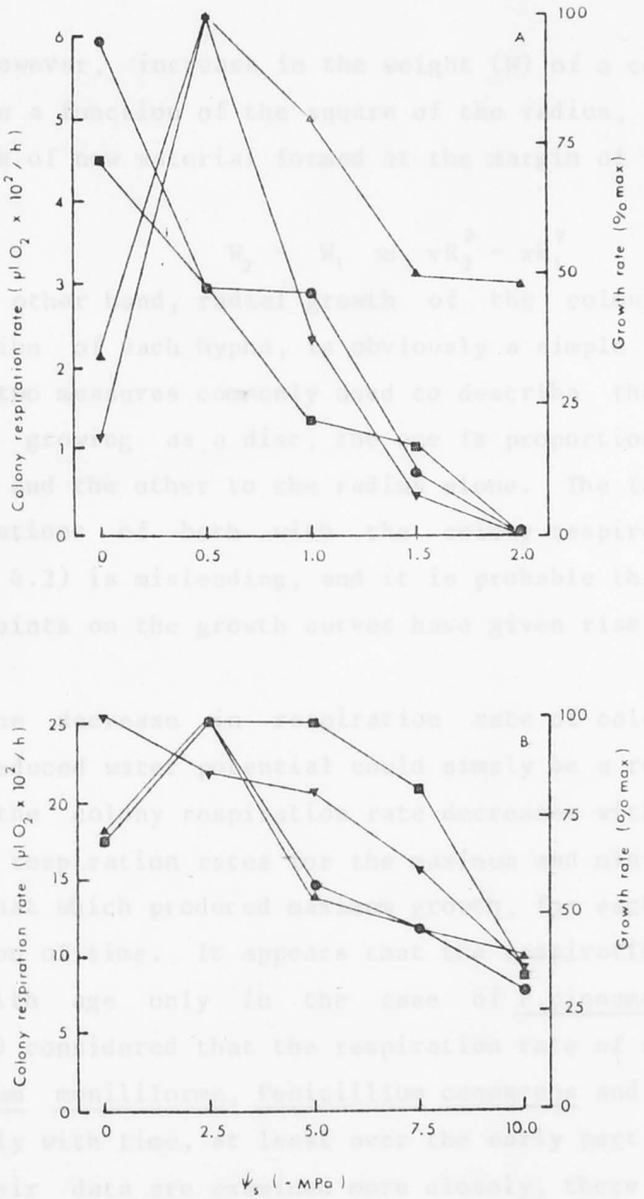


Fig. 4.2. Effect of water potential on steady state growth and respiration rates in Phytophthora cinnamomi (A) and Penicillium chrysogenum (B) grown on KCl. Respiration rates of colonies of the same age (●) and of the same radius (■) and growth rates, both radial (▲) and increase in dry weight (▼) are shown.

However, increase in the weight (W) of a colony, on a daily basis, must be a function of the square of the radius, if it assumed that the annulus of new material formed at the margin of the colony is of minimal depth:

$$W_2 - W_1 \approx \pi R_2^2 - \pi R_1^2$$

On the other hand, radial growth of the colony, due to the linear extension of each hypha, is obviously a simple function of R. Thus, of these two measures commonly used to describe the growth of a fungal colony growing as a disc, the one is proportional to the square of the radius and the other to the radius alone. The fact that very similar correlations of both with the colony respiration rate were obtained (Table 4.2) is misleading, and it is probable that the small number of data points on the growth curves have given rise to this result.

The decrease in respiration rate of colonies of the same radius with reduced water potential could simply be a reflection of the fact that the colony respiration rate decreases with age. In Fig. 4.3, the colony respiration rates for the maximum and minimum potentials tested and that which produced maximum growth, for each species are shown as a function of time. It appears that the respiration rate certainly falls off with age only in the case of P.cinnamomi. Wilson and Griffin (1975b) considered that the respiration rate of colonies of P.cinnamomi, Fusarium moniliforme, Penicillium canescens and Geastrum sp. increased linearly with time, at least over the early part of the curve. However, if their data are examined more closely, there was probably a decrease in the respiration rate in all four of their species at the end of the experimental period (6 days), although at the higher of the two potentials tested only. The radial growth rate of fungal colonies does not appear to decrease with time, at least when colonies are grown on 9 cm Petri plates (Chapter 2; Wilson & Griffin, 1975b). These considerations suggest that the decrease in colony respiration rate at low potential was probably not simply the result of age.

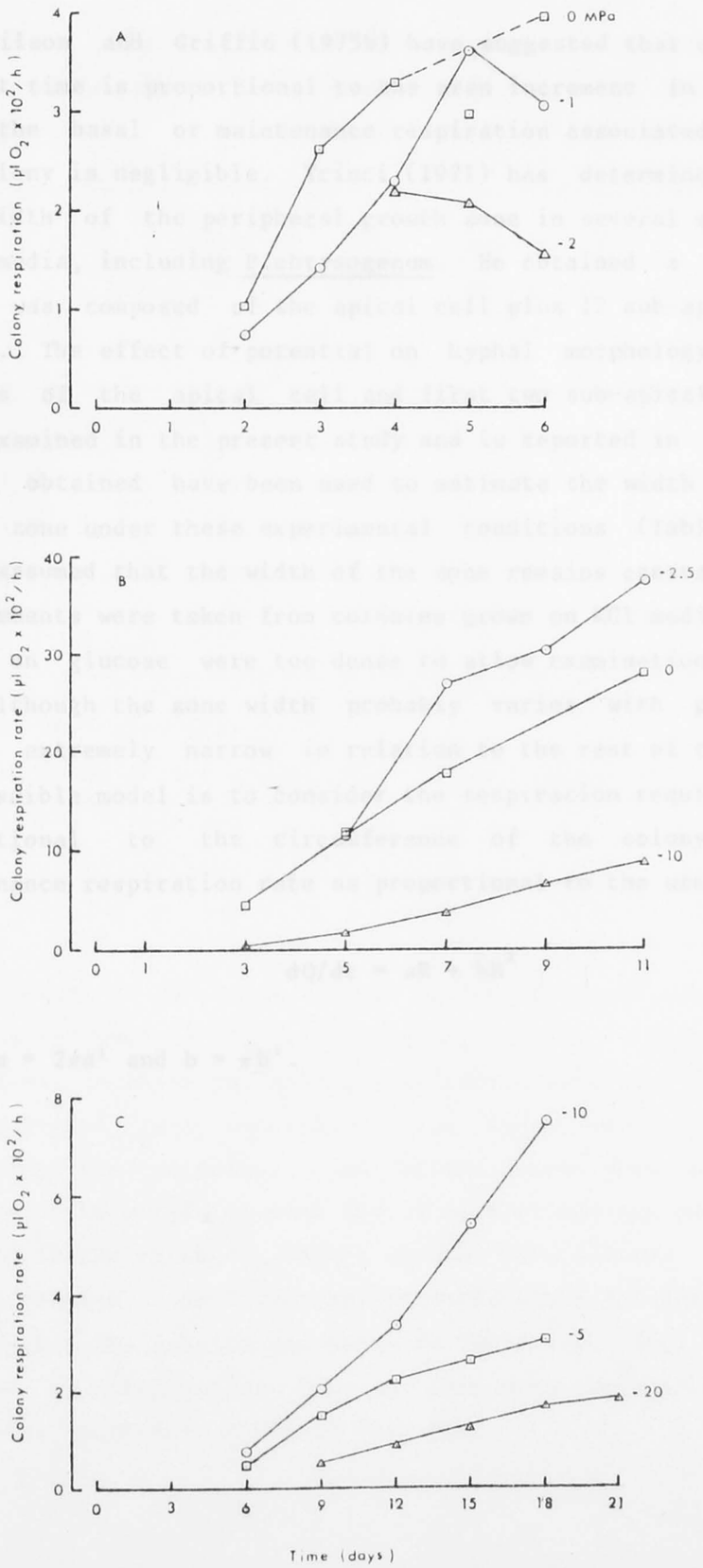


Fig. 4.3. Change in respiration rate with colony age in Phytophthora cinnamomi (A), Penicillium chrysogenum (B) and Chrysosporium fastidium (C) grown at the highest (□) and lowest (△) potentials tested and that which produced the maximum growth rate (○).

Wilson and Griffin (1975b) have suggested that colony respiration in unit time is proportional to the area increment in that time, and that the basal or maintenance respiration associated with the rest of the colony is negligible. Trinci (1971) has determined experimentally the width of the peripheral growth zone in several species growing on solid media, including P.chrysogenum. He obtained a value of 496 μm which was composed of the apical cell plus 12 sub-apical compartments behind. The effect of potential on hyphal morphology, including the lengths of the apical cell and first two sub-apical compartments has been examined in the present study and is reported in Chapter 5. The values obtained have been used to estimate the width of the peripheral growth zone under these experimental conditions (Table 4.3). It has been assumed that the width of the zone remains constant with age. The measurements were taken from colonies grown on KCl media because hyphae grown on glucose were too dense to allow examination. It can be seen that although the zone width probably varies with potential, it is always extremely narrow in relation to the rest of the colony so that one possible model is to consider the respiration required for growth as proportional to the circumference of the colony, and basal or maintenance respiration rate as proportional to the area:

$$dQ/dt = aR + bR^2$$

where $a = 2\pi a'$ and $b = \pi b'$.

Table 4.3. The effect of water potential produced by the addition of KCl on the width of the peripheral growth zone in Penicillium chrysogenum.

ψ_s (-MPa)	Apical cell* (μm)	Sub-apical compartment* (μm)	Peripheral growth zone** (μm)
0	274	26	586
2.5	291	31	663
5.0	288	34.5	702
7.5	136	25	436
10.0	88	21	340

* Data taken from Chapter 5.

** Calculated as the apical cell + 12 sub-apical compartments (Trinci, 1971).

In order to determine whether or not the basal respiration of the non-growing part of the colony was significant, a single experiment was carried out in which the hourly respiration rate of 9 day old -2.5 MPa KCl colonies was determined from three replicate chambers each containing five colonies. The plates were then removed from the chambers, the margin (1 to 2 mm) of each colony cut off, and the plates replaced in the chambers. Ninty minutes were allowed for healing and reequilibration. The new respiration rate was determined over a period of 2 hours. The results are shown in Table 4.4. The rate started to increase in the second hour so that some regrowth of the margin had presumably started to occur by this time.

Table 4.4. Respiration of the margin and interior of 9 day old Penicillium chrysogenum colonies of radius 26 mm grown on -2.5 MPa KCl. Values are the means and standard deviations from three respirometer chambers.

Parameter	Whole colony	Interior	Margin*
µl/total area/h	2416±191	1391±22	1025±211
µl/mg fw/h	3.0±0.2	1.8±0.1	26.4±4.4
µl/mg dw/h	10.8±1.0	6.5±0.2	100.7±19.6
µl/mm^2 area/h		0.66	
µl/mm circumference/h			6.27

* Values obtained by subtraction of interior from whole colony.

From this experiment, the following relationship for the respiration rate of the whole colony can be established:

$$dQ/dt = 39.40R + 2.07R^2$$

where the unit of dQ/dt is $\mu l O_2$/colony/h and of R is mm. When theoretical respiration rates at other potentials are calculated from this equation using the measured radii, they appear to correlate reasonably well with the actual colony rates measured at all potentials and ages for the KCl series, except possibly the 7 and 9 day old colonies (Fig. 4.4). The estimates of these two coefficients obtained by fitting an $(aR + bR^2)$ relationship to the data by the method of least squares using GLIM statistical package (Nelder, 1975) were 73.81 and 1.18 respectively. However neither of these were significantly different from zero (P = 0.05) owing to the small number of values.

Alternatively, the assumption can be made that when the radius is small, the basal contribution approximates to zero. Thus the rate per

unit of circumference can be calculated from the early measurements and used to determine the relative contributions of circumference and basal region for the rest of the period, for each potential. The coefficients obtained are given in Table 4.5.

Table 4.5. Coefficients for the equation $dQ/dt = aR + bR^2$.

ψ_s (-MPa)	a *	b			
	day 3-11	day 7	day 9	day 11	mean
0	12.8	0.20	-	0.10	0.15
2.5	12.2	0.93	0.42	0.30	0.55
5.0	11.4	0.83	0.83	0.37	0.68
7.5	13.4	0.43	0.43	0.23	0.36
10.0	7.7	1.47	1.47	0.83	1.26

* Based on rates from days 3 and 5, assuming a peripheral growth zone 1.5 mm wide.

The values for the a coefficient were similar for the first four potentials, but it was considerably less for the colonies growing at -10 MPa. This suggests a decrease in the respiration rate per unit of growth only at the lowest potential. The values for the b coefficient were lowest from 0 MPa colonies, similar for the next thee potentials and considerably higher at -10 MPa, implying a greater contribution of maintenance respiration at this lowest potential. Unfortunately there were insufficient data to justify statistical comparison of the coefficients but the implication is that P.chrysogenum is well adjusted to this range of potential, with the possible exception of the lowest potential tested. Although the width of the growth zone varied considerably between species and was greatest for the coenocytic species (Trinci, 1971), this assumption probably holds for all three

experimental species.

The experiment also demonstrates that although the unit respiration rate of the interior is an order of magnitude less than that of the margin (Table 4.2), it is by no means insignificant. Large colonies will therefore have a considerable maintenance requirement for oxygen which is not associated with growth. Conidiation of course occurs on the non-growing part of Penicillium colonies and will have a specific energy and oxygen requirement which will be included in the maintenance fraction as calculated here.

However another model might be to consider that a reduction in supply of nutrients to the interior of the colony, or progressive death of older parts, in addition to continued radial growth at the margin, account for the observed decrease in the colony respiration rate with time. If this were the case, a logistic function might be a more valid expression for Q than that given above. This is a function sometimes used to express population increase and may be considered as describing both a continual growth process and continual death. The curve is sigmoid and incorporates a lag phase, then a linear phase and finally approaches an asymptote:

$$Q = f(t) = X_e / \left[1 + \exp(-CX_e(t + B))\right]$$

rearranged from the equation of Maynard Smith (1968) where X_e is the asymptote, CX_e the rate of increase when Q is small and B an arbitrary constant which can be chosen to fit the initial conditions.

Although the fitted logistic function did reduce the variation as compared to the $(aR + bR^2)$ relationship, the improvement using the F test was not significant because of the paucity of the data (Table 4.6). The same result was obtained at all the other potentials except 0 MPa KCl where the $(aR + bR^2)$ relationship was the less variable. Clearly more data are needed to analyse these relatioships statistically and the best way of determining if a logistic relationship is applicable would be to grow large colonies in for instance 14 cm diameter petri plates but with the same depth of agar as the small plates, and establish if the respiration rate approaches an asymptote. Then also the estimates

of the parameters would be a valuable guide to the effect of potential on respiration.

Table 4.6. Test of significance of departure of Q = f(t) from Q = ($aR + bR^2$) regression at -2.5 MPa KCl.

Source of variation	Degrees of freedom	Sums of squares	Mean square
Deviations from ($aR + bR^2$)	2	138400	
Deviations from logistic*	1	110453	110453
Logistic relationship	1	27947	27947 Not significant
	F = 27947/110453 = 0.25		

* Fitted using NLPE computer programme (Bard, 1967).

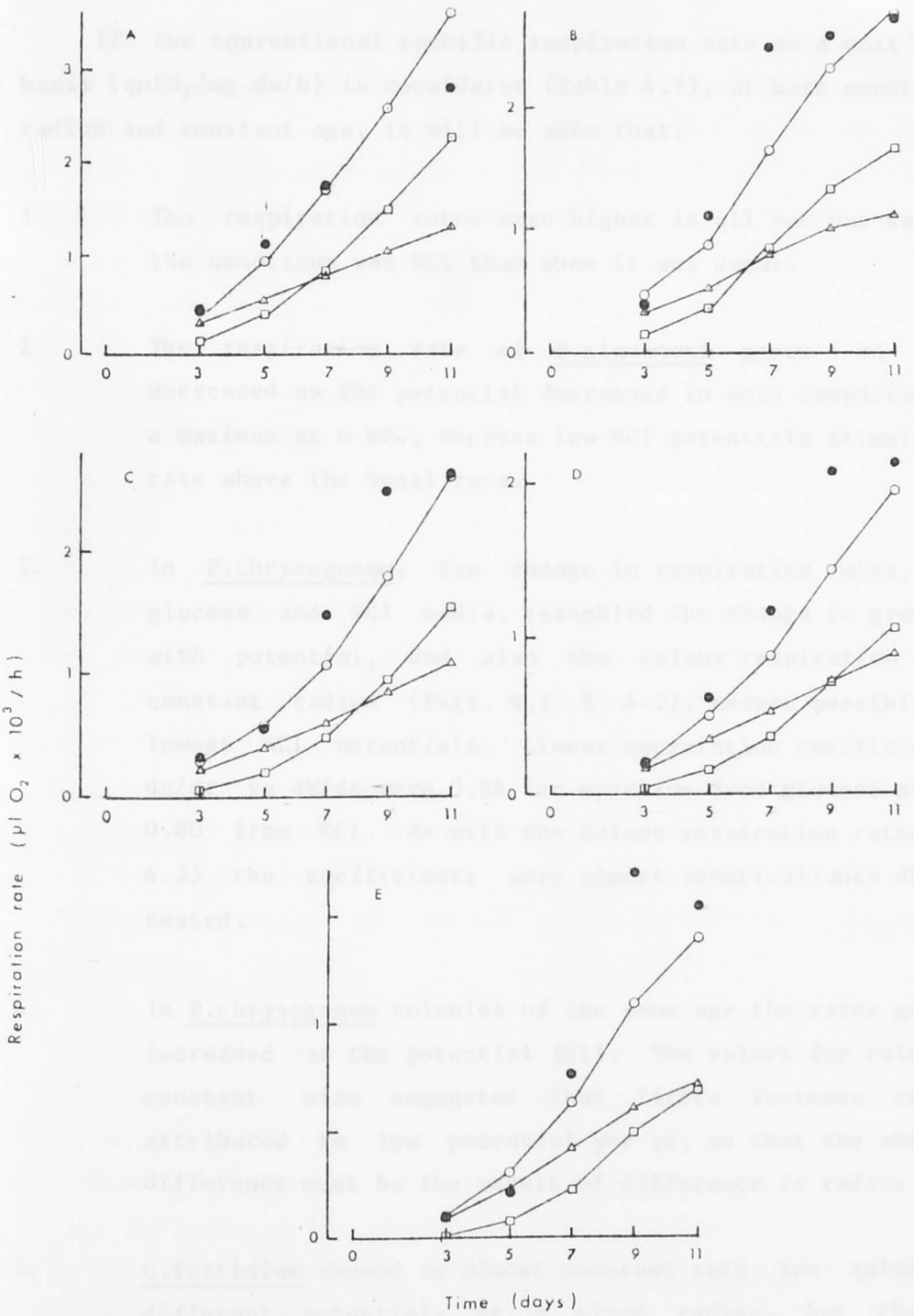


Fig. 4.4. Change in calculated maintenance (□), growth zone (△) and total (○) respiration rates and observed total respiration rate (●) with time in Penicillium chrysogenum grown on KCl osmotica of 0 MPa (A), -2.5 MPa (B), -5 MPa (C), -7.5 MPa (D) and -10 MPa (E). See text for method of calculation.

If the conventional specific respiration rate on a unit dry weight basis ($q\mu lO_2$/mg dw/h) is considered (Table 4.7), at both constant colony radius and constant age, it will be seen that:

1. The respiration rates were higher in all but one cases when the osmoticum was KCl than when it was sugar.

2. The respiration rate of P.cinnamomi grown on sucrose decreased as the potential decreased in both comparisons from a maximum at 0 MPa, whereas low KCl potentials stimulated the rate above the basal rate.

3. In P.chrysogenum, the change in respiration rates, on both glucose and KCl media, resembled the change in growth rate with potential, and also the colony respiration rates at constant radius (Figs. 4.1 & 4.2), except possibly at the lowest KCl potentials. Linear correlation coefficients for dq/dt vs dW/dt were 0.96 for mycelium from glucose media and 0.80 from KCl. As with the colony respiration rates (Table 4.2) the coefficients were almost identical when dR/dt was tested.

4. In P.chrysogenum colonies of the same age the rates generally increased as the potential fell. The values for colonies at constant size suggested that little increase could be attributed to low potential per se, so that the additional difference must be the result of difference in radius (size).

5. C.fastidium showed an almost constant rate for colonies of different potentials at a given radius, but the rate decreased by almost half at the lowest potential tested.

6. In colonies of C.fastidium of the same age, the rates again were very similar for all the potentials except the two lowest, where fructose as well as glucose was present in the medium. There may also have been a slight increase in growth rate at these two potentials (Fig. 4.1).

However, it is doubtful whether a specific respiration rate is a valid measure of comparison in a study such as this. It has been demonstrated in the previous chapter that growth at low water potential results in the accumulation of a considerable weight of solutes. In the absence of information on the effect of potential on the distribution or numbers of organelles, it may be assumed that accumulated sugars and polyols 'dilute' the cytoplasmic material necessary for biosynthesis (see Chapter 5). If a correction is made for the accumulated osmoregulatory solutes, using the information presented in Fig. 3.3B and Fig. 3.4A for P.chrysogenum, then it can be seen that there is an increase in specific respiration rate for colonies of constant radius (Table 4.7, figures in brackets). There was therefore even less inhibition of the respiration rate by low water potential. The correlation coefficients for these adjusted specific respiration rates with growth rate were consequently reduced, most noticeably in the KCl series (0.87 and 0.51 for growth on glucose and KCl media respectively). However it is interesting that when the results are considered on a unit weight basis, the extremely high rate of respiration observed for P.chrysogenum colonies disappears and the rates for all three species are of the same order, although P.cinnamomi was the lowest. Rates at an equivalent potential of -10 MPa were similar in P.chrysogenum and C.fastidium.

The specific respiration rate was also considered as a function of time (Fig. 4.5) in the same manner as the colony respiration rate (Fig. 4.3). It will be seen from the former that the change with age in the specific respiration rate (dq/dt) is very similar to the change with age in the ratio of the peripheral growth zone or circumference to the rest of the colony area (Fig. 4.5). The following deductions can be made from this graphical correlation:

$$\text{circumference:area} \approx 1/R$$

therefore:

$$dq/dt \approx 1/R$$

The respiration rate of the whole colony (dQ/dt) can be obtained by multiplying the specific rate by the weight of the colony:

$$dQ/dt = dq/dt \times W(col)$$

As discussed earlier, the depth of the colony can be considered to be negligible so that:

$$W(col) \approx \pi R^2$$

Thus the change in colony respiration rate with time will be:

$$(dQ_1/dt)/(dQ_2)/dt) \approx (R_2 x\ \pi R_1^2\ )/(R_1 x \pi R_2^2\ )$$
$$\approx R_1/R_2$$

Previously a relationship of colony respiration rate proportional to $R + R^2$ was considered. The above treatment confirms the finding of the importance of the respiration attributable to the growing margin (R) relative to the basal respiration of the remainder of the colony (R^2) (Table 4.4).

Table 4.7. The respiration rates on a dry weight basis of P.cinnamomi, P.chrysogenum and C.fastidium grown on different potentials and osmotica comparing colonies of equal radius and of equal age.

Species	ψ_s (-MPa)	Rate at r=21±2 mm ($\mu l O_2$/mg dw/h)		Rate at t=3* ($\mu l O_2$/mg dw/h)	
P.cinnamomi		sucrose	KCl	sucrose	KCl
	0	7.44	8.17	12.47	8.17
	0.5	6.22	7.03	6.75	-
	1.0	5.81	9.72	6.45	10.91
	1.5	4.71	18.10	6.89	-
	2.0	3.26	12.76**	8.71	-
P.chrysogenum		glucose	KCl	glucose	KCl
	0	12.81	12.51	12.81	12.81
	2.5	15.07(16.6)	20.17(23.2)	15.07	20.17
	5.0	13.85(16.1)	17.70(21.8)	13.85	20.47
	7.5	12.11(14.5)	11.70(15.3)	15.07	22.76
	10.0	9.74(12.1)	14.51(20.0)	19.26	29.53
C.fastidium		glucose		glucose	
	5.0	7.73**		10.36	
	7.5	11.03		13.01	
	10.0	11.70		12.67	
	12.5	12.30		13.09	
	15.0	12.27		12.12	
	17.5†	12.94		19.09	
	20.0†	7.48		16.93	

* P.cinnamomi 4 days, P.chrysogenum 7 days and C.fastidium 12 days.

** Colony less than 21 mm.

† glucose + fructose

( ) dry weight adjusted for polyol content.

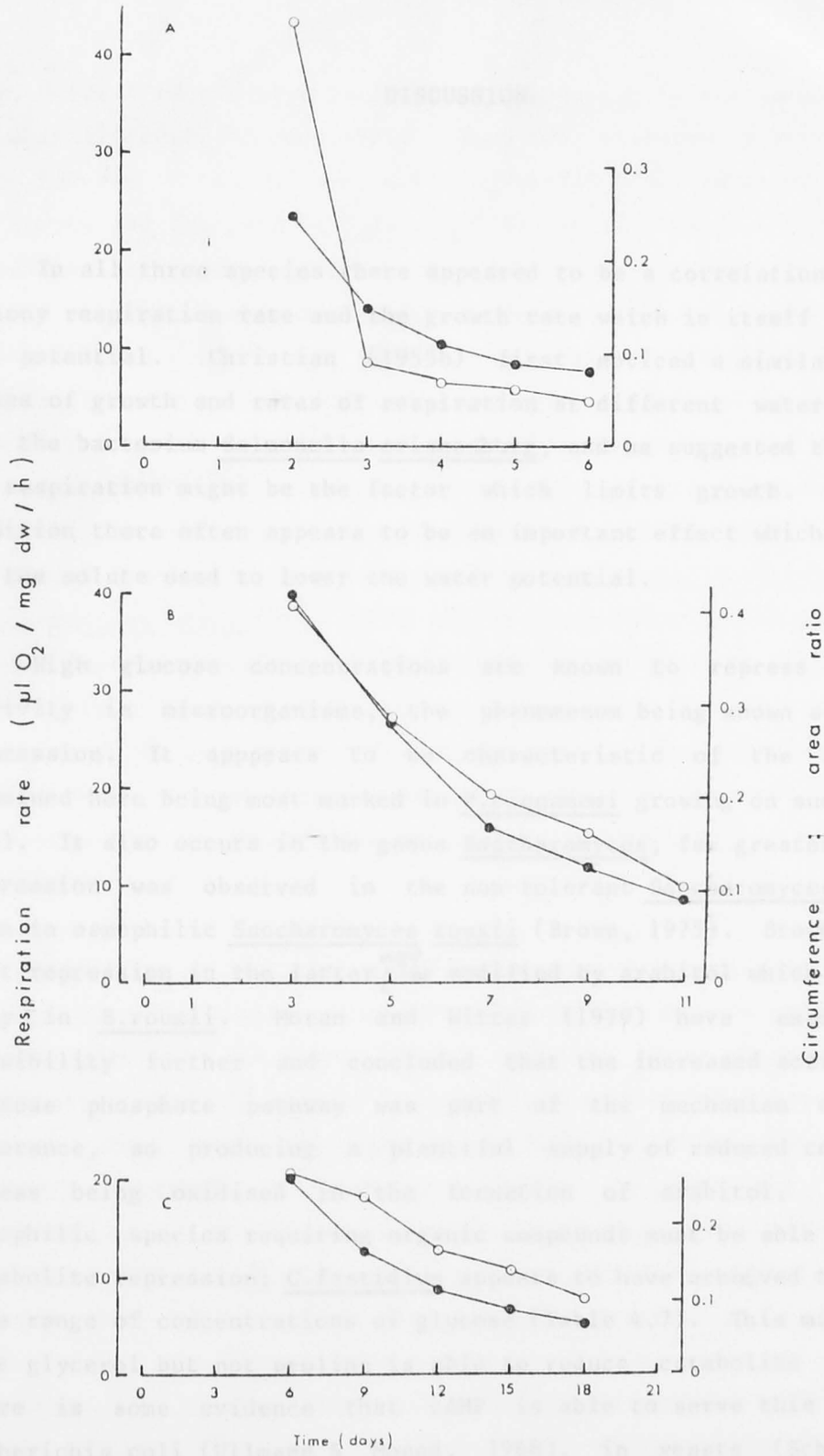


Fig. 4.5. Change in specific respiration rate (○) and in circumference to area ratio (●) with time in Phytophthora cinnamomi grown on -1 MPa sucrose (A), Penicillium chrysogenum on -10 MPa glucose (B) and Chrysosporium fastidium on -10 MPa glucose (C).

DISCUSSION

In all three species there appeared to be a correlation between the colony respiration rate and the growth rate which is itself a function of potential. Christian (1955b) first noticed a similarity between rates of growth and rates of respiration at different water potentials in the bacterium Salmonella orianenburg, and he suggested that the rate of respiration might be the factor which limits growth. However in addition there often appears to be an important effect which is specific to the solute used to lower the water potential.

High glucose concentrations are known to repress respiratory activity in microorganisms, the phenomenom being known as catabolite repression. It apppears to be characteristic of the three fungi examined here being most marked in P.cinnamomi growing on sucrose (Table 4.7). It also occurs in the genus Saccharomyces; far greater catabolite repression was observed in the non-tolerant Saccharomyces cerevisiae than in osmophilic Saccharomyces rouxii (Brown, 1975). Brown suggested that repression in the latter might be modified by arabitol which accumulated only in S.rouxii. Moran and Witter (1979) have examined this possibility further and concluded that the increased activity of the pentose phosphate pathway was part of the mechanism of glucose tolerance, so producing a plentiful supply of reduced coenzyme, any excess being oxidised in the formation of arabitol. Obligately osmophilic species requiring organic compounds must be able to overcome catabolite repression; C.fastidium appears to have achieved this over a wide range of concentrations of glucose (Table 4.7). This might suggest that glycerol but not proline is able to reduce catabolite repression. There is some evidence that cAMP is able to serve this function in Escherichia coli (Ullmann & Monod, 1968), in yeasts (Schlanderer & Dellweg, 1974) and also in Dendryphiella salina (Galpin et al., 1977).

The effect of potential on respiration on a number of yeasts has been examined by other workers, and as with the filamentous fungi studied here, the results were fairly specific to the species. S.rouxii

had a lower respiration rate than S.cerevisiae in the basal (high water potential) medium (Brown, 1975). When the reaction mixture potential was lowered with PEG to 0.95 a_w (-7.1 MPa), rates of both species decreased and the rate of S.rouxii was still lower. Growth in PEG increased the subsequent respiration rate in PEG of both species. Growth in glucose also decreased the respiration rate of S.rouxii by about 20% and S.cerevisiae by 75%, but within the range of 24 to 48% w/v glucose (ca -4 to -10 MPa) there was no difference in effect. The specific respiration rate of S.cerevisae decreased also when glycerol was added to the growth medium; 5 to 10% glycerol (-1.4 to -3 MPa) resulted in a 40% inhibition (Benllock & de Castro, 1979). Three other species did not give this result and in Saccharomyces carlsbergensis and Saccharomyces oviformis there was an enhancement at 10% over the basal rate. Repressed cells grown on 10% glucose were able to adapt to a higher rate when transferred to dilute media, but this did not occur when the cells were repressed with glycerol, thus indicating genetic differences and not lack of enzymes catabolising glycerol. The results suggested that glycerol had a detrimental effect on the biogenesis of mitochondria.

Salt however does not appear to inhibit respiration in the same way as sugars. KCl usually stimulated respiration rates in relation to the growth rate in the three fungi used in these experiments. The respiration rates of several species of marine yeasts were similar without added NaCl and with 4% w/v NaCl (-3.2 MPa), but decreased at higher concentrations (Norkrans, 1968). As with the experiments reported here, there was a solute specific effect. The minimum a_w permitting 10% respiration activity was higher for electrolytes than for glucose and lowest of all for glycerol. Respiration of an obligately marine fungus Thraustochytrium roseum was stimulated by the addition of a number of solutes, both electrolytes and non-electrolytes at about -1 MPa compared with rates in dilute media (Siegenthaler, et al., 1967). Low water potentials (min -5 MPa KCl) decreased both total respiration and the rate of respiration in conidia of Verticillium albo-atrum (Mozumder & Caroselli, 1970). Reduction of growth and respiration in sea water were similar in a fresh water Ascomycete (Davidson, 1974).

Effects specific to salt have been observed in other organisms.

NaCl up to 0.8 molar (-3.7 MPa) stimulated oxidation of succinate in resting cells of a moderately halophilic bacterium, but KCl and sucrose did not. Higher NaCl concentrations had an inhibitory effect (Rafaeli-Eshkol, 1968). Growth rate and respiration both declined with lowered water potential in another baterium Pseudomonas fluorescens (Prior, 1978). He noted effects specific to the solute; NaCl was most inhibitory, then sucrose and glycerol the least of all. Growth also occurred at the lowest potential when glycerol was used.

In the green alga Chlorella pyrenoidosa the inhibitory effect of low water potential on the dark respiration rate was associated with reduced uptake of substrate, either glucose or acetate, rather than reduction of the activity of the respiratory pathway (Greenway & Hiller, 1967). In this case solute specific differences were not observed (mannitol, KCl, glucose, PEG) so that the effect was attributed to water potential per se. Thus the respiration rate in a number of microorganisms appears to be a complex function of both potential and solute which is species specific.

The concept (Wilson & Griffin, 1975b) that growth at low water potential is a necessarily inefficient process that has a high energy demand is probably an oversimplification. They compared two potentials only and used one osmoticum (KCl). Their results suggested that the colony respiration rate per unit growth was 10.88 times greater for P.cinnamomi growing at -4.1 MPa than at -0.1 MPa. The three other species they compared were F.moniliforme, P.canescens and Geastrum sp. from -0.1 MPa and -8.1 MPa. For F.moniliforme also, the rate was appreciably higher at the lower potential (x 6.23) but for P.canescens and Geastrum sp. the difference was probably not significant (x 1.43 and 2.10 respectively). Gustaffson, working on the marine yeast Debaryomyces hansenii, also used a salt, NaCl, as the osmoticum and she found that efficiency was reduced by low potential. In this case she used heat production as a measure of activity and observed that the molar growth yield was lower when the yeast was grown at -2.7 molar NaCl (-14.6 MPa) than at 0.004 molar (Gustaffson & Norkrans, 1976; Gustaffson, 1979). However in another yeast, Candida utilis, increasing the NaCl concentration to 20 g/l (-1.6 MPa) had no effect on the molar growth yield or on the specific respiration rate (Aiking et al., 1977).

It is clear that generalisations on the effect of potential cannot be made from a comparison of two potentials only, and that the type of solute also must be considered. However, the data from my more extensive experiments, also are not conclusive. When specific respiration rates for colonies of P.chrysogenum of the same radius were compared, respiration rate and growth rate seemed well correlated (Table 4.7). When an adjustment for the accumulated solutes (glycerol and glucose or KCl) was made to the dry weight, this correlation was less obvious. Instead, at least in the KCl series, the respiration rate was approximately constant and double that of colonies from the highest water potential. The effect of glucose on the respiration rate of this species was different to that of KCl although the repression from glucose at low potential was less marked when the dry weights were adjusted. There was some evidence of enhancement of specific respiration rates of P.cinnamomi by growth at low KCl potentials (Table 4.7), but not to the extent of that noted by Wilson and Griffin (1975b). Of the sugars, only fructose had a stimulatory effect, and this was on C.fastidium (Table 4.7). This was probably not due to potential but related to its stimulation of growth.

The only other known estimate of the maintenance respiration requirement appears to be that of Righelato et al. (1968). Also working on P.chrysogenum, they used a chemostat culture and reduced the glucose concentration until growth just ceased and thus determined the oxygen and glucose uptake required for maintenance. The values obtained were 0.12 millimoles glucose/g dw/h and 0.74 millimoles O_2/g dw/h. They also demonstrated that this maintenance ration was important for preventing autolysis and facilitating conidiation.

Growth of filamentous fungi in unlimited liquid culture is a function of α, the specific growth rate, which is considered to be an intrinsic characteristic of each species:

$$dW/dt = \alpha W$$

which on integration becomes:

$$\ln W_2 - \ln W_1 = \alpha t$$

Trinci (1969) has shown that this characteristic can be related to the linear phase of radial growth of a colony of that species although the relationship cannot be used when the concentration of nutrients is varied:

$$R_2 = R_1 + K_r(t_2 - t_1)$$

where K_r is a constant. The ratio K_r/α remained constant for growth of Aspergillus nidulans at different temperatures (i.e. at different growth rates), although Sterne and McCarver (1978) claimed that there was not a constant relationship between K_r and α for Rhizoctonia solani, Pythium ultimum or Verticillium dahliae grown at different osmotic potentials. This constant for P.chrysogenum was 432. The linear radial growth rate of P.chrysogenum growing on -2.5 MPa KCl was 2.78 mm/24 h or 116 μm/h. If the constant (K_r/α) of Trinci is used to calculate α, a value of 0.27 is obtained. This was considerably higher than any of the observed chemostat growth rates, but by extrapolating from the relationship between respiration and growth rate of Righelato et al. a respiration rate of about 6 millimoles O_2/g dw/h is obtained, and on a dry weight basis, the maintenance ration was 12%. This compares reasonably with the values of 6% on a dry weight basis, or 11% on a unit area basis (Table 4.4) obtained in these experiments.

THE EFFECT OF OSMOTIC SHOCK ON RESPIRATION

Colonies of suitable size were placed in respiration chambers, allowed to equilibrate and a steady state respiration rate obtained after 1 to 2 hours. The plates were then removed from the chambers and the colonies placed on fresh plates as described in Chapter 3. The process, including reequilibration, took about 30 min so that the initial rates observed on the new plates may have been artificially low. Respiration on the new media was followed for several hours, or for up to 3 days in the case of C.fastidium. Shock experiments were performed as follows:

P.cinnamomi	2 KCl → 0 KCl (-MPa)
P.chrysogenum	10 glucose → 0 glucose
C.fastidium	10 glucose → 0 glucose
P.cinnamomi	0 KCl → 2 KCl
P.chrysogenum	0 glucose → 10 glucose
C.fastidium	10 glucose → 20 glucose
C.fastidium	10 glucose → 10 KCl

RESULTS

Following hyperosmotic shock, the rate of respiration on a colony basis increased in all three species (Fig. 4.6). The steady state rate for colonies of the same size on the new potential are shown, and it will be seen that these are lower than the initial rates on the high

water potential media, so that a decrease in rate might be expected. There was some evidence that this had started to occur in P.cinnamomi after 5 h at -2 MPa KCl. The increased respiration rates observed presumably therefore reflect the synthesis or accumulation of solutes to adjust to the new potential which was shown in Chapter 3 to take several hours.

Hypoosmotic shock also produced an increase in the colony respiration rate as might be expected. The new steady state had been reached in P.cinnamomi after about 6 h. The P.chrysogenum rate again exceeded the steady state rate on 0 MPa glucose after 6 h. This may have been due to oxidative respiration of the additional carbohydrate within the hyphae, or reflect an energy requirement for conversion of glycerol to insoluble polysaccharide, or to use the terminology of Gustaffson (1979), the reorganisation of the cellular structure to a water ordered and not a glycerol ordered state.

The rate of respiration of C.fastidium decreased following hypoosmotic shock (the initial increase probably reflects lack of equilibrium in the chambers). The decline in the rate was fairly slow so that death does not appear to have been instantaneous. In fact a measurable rate was maintained after 2 days.

When C.fastidium was transferred to isoosmotic KCl, a steady rate of respiration was maintained for at least 3 days at a value somewhat higher than the glucose rate. However no growth was observed on control colonies during this period, or after 1 week. The tips did not burst and brown pigment was released into the medium beneath the colony in the usual way. Growth resumed after several days when the colonies were transferred back to glucose media, thus the inhibition was not lethal.

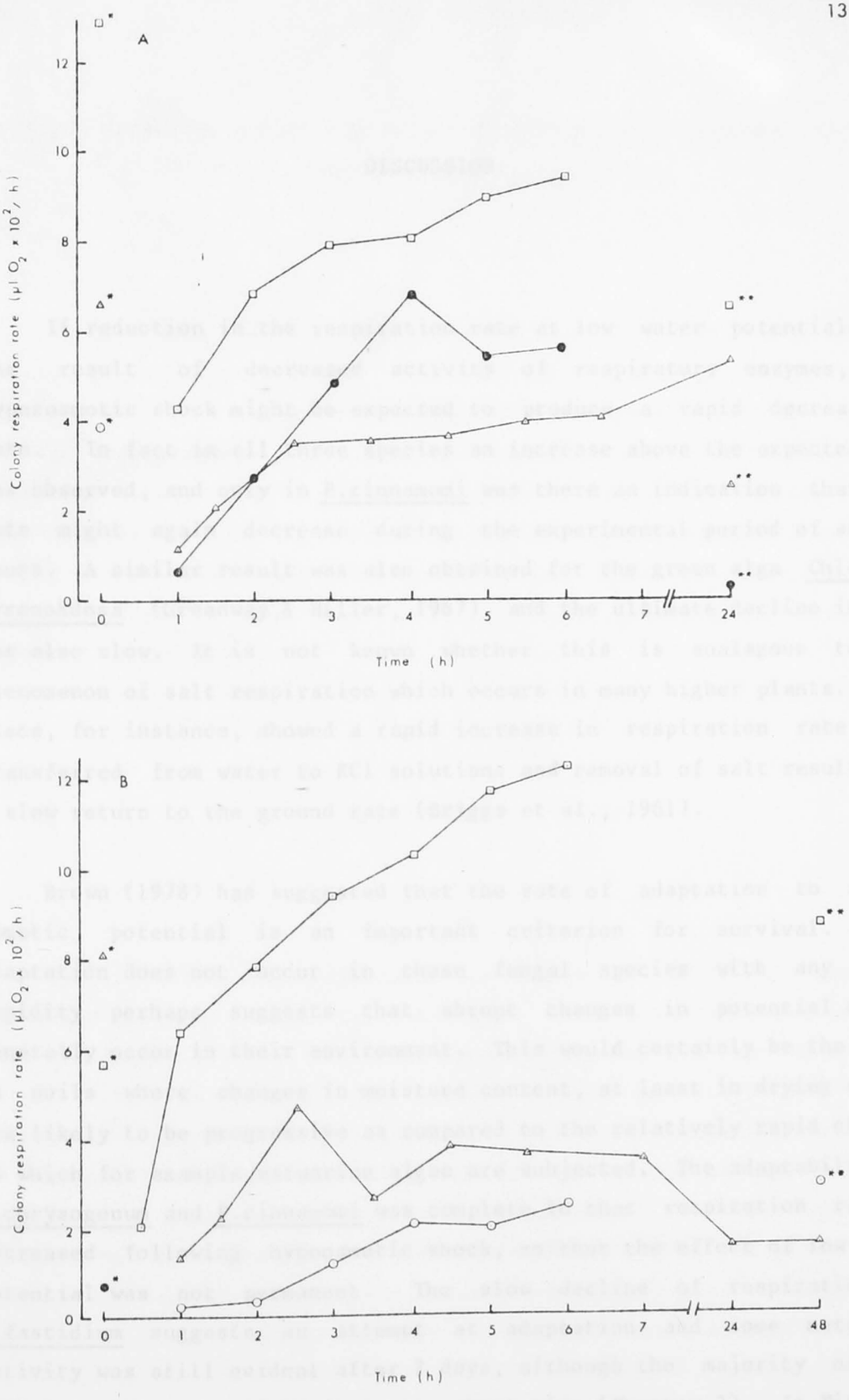


Fig. 4.6. Effect of hyperosmotic shock (A) and hypoosmotic shock (B) on colony respiration rate of Phytophthora cinnamomi (○,●), Penicillium chrysogenum (□) and Chrysosporium fastidium (△). KCl osmotica shaded symbols, sugar osmoticum open symbols. * Initial steady state rate, ** steady state rate for colony of same radius at the new potential. Values are means from three chambers. NB. B□ respiration scale = x 0.5.

DISCUSSION

If reduction in the respiration rate at low water potentials was the result of decreased activity of respiratory enzymes, then hyperosmotic shock might be expected to produce a rapid decrease in rate. In fact in all three species an increase above the expected rate was observed, and only in P.cinnamomi was there an indication that the rate might again decrease during the experimental period of several hours. A similar result was also obtained for the green alga Chlorella pyrenoidosa (Greenway & Hiller, 1967), and the ultimate decline in rate was also slow. It is not known whether this is analagous to the phenomenon of salt respiration which occurs in many higher plants. Beet discs, for instance, showed a rapid increase in respiration rate when transferred from water to KCl solutions and removal of salt resulted in a slow return to the ground rate (Briggs et al., 1961).

Brown (1978) has suggested that the rate of adaptation to a new osmotic potential is an important criterion for survival. That adaptation does not occur in these fungal species with any great rapidity perhaps suggests that abrupt changes in potential do not generally occur in their environment. This would certainly be the case in soils where changes in moisture content, at least in drying soils, are likely to be progressive as compared to the relatively rapid changes to which for example, estuarine algae are subjected. The adaptability of P.chrysogenum and P.cinnamomi was complete in that respiration rapidly increased following hypoosmotic shock, so that the effect of low water potential was not permanent. The slow decline of respiration in C.fastidium suggests an attempt at adaptation and some metabolic activity was still evident after 2 days, although the majority of the hyphal solutes were lost in a very short time (Chapter 3). In Nitella, a giant alga, apparently prolonged hyperosmotic shock resulted in a fall in the threshold value for extensibility and growth to permit resumption of growth at reduced turgor (Green, 1968). The effects of shock on growth have been examined in more detail in Chapter 5.

THE EFFECT OF GROWTH CONDITIONS OF PHYTOPHTHORA CINNAMOMI ON ISOCITRATE DEHYDROGENASE ACTIVITY

Phytophthora cinnamomi was grown on 20 plates of each of the following potentials: 0 MPa, -1 MPa sucrose, -1 MPa KCl, -2 MPa sucrose and -2 MPa KCl, on cellophane as described previously. A crude extract of each was prepared, and isocitrate dehydrogenase activity assessed at different potentials. The potential of 62.5 millimolar bicine buffer was -0.2 MPa, so that to bring it to the same actual potential as 0 CYA, 30 g/l sucrose was added. Additional sucrose or KCl was added to bring the potential to the same as those of the growth media used in the previous experiments.

RESULTS

The results of this experiment are shown in Table 4.8, and are expressed in terms of relative inhibition. Two conclusions can be drawn from the data. Firstly, the conditions of growth did not appear to affect the response of the enzyme extract to different osmotic potentials. Enzyme from hyphae grown at the lowest potential was just as susceptible to inhibition by both KCl and sucrose as that extracted from material grown at the highest water potential. Nor was there any enhancement of enzyme activity from growth at -1 MPa, the potential at which other factors have been stimulated (Chapters 2 & 3). However,two way analyses of variance, of both the series assayed with KCl and that assayed with sucrose, indicated that the conditions of growth did not alter significantly (P=0.05) the activity of the enzyme, in the former series only.

Secondly, it can be seen that there was a solute specific effect in that sucrose was considerably more inhibitory than KCl, the maximum inhibition by the former being about twice that by the latter. The

repressive effect of sucrose appeared to be a function of concentration since the minimum inhibition occurred at 0 MPa which contained least sucrose. In the case of KCl however, minimum inhibition was observed at -0.5 MPa in all but one of the extracts. This response therefore resembled the growth response curves previously reported. The effects of different solutes on enzyme activity will be examined in more detail in the following experiment.

Table 4.8. The effect of the conditions of growth on isocitrate dehydrogenase extracted from P.cinnamomi assayed at different potentials produced with sucrose or KCl osmotica.

	Enzyme inhibition (%)									
ψ_s (assay)	ψ_s (growth)									
(-MPa)	0 MPa		-1 MPa sucrose		-1 MPa KCl		-2 MPa sucrose		-2MPa KCl	
	S	K	S	K	S	K	S	K	S	K
0	0	5	0	19	0	2	0	11	0	0
0.5	20	0	11	0	23	0	11	0	5	4
1.0	35	0	20	7	40	8	20	4	21	-
1.5	45	14	36	21	45	21	33	20	34	17
2.0	55	24	46	25	51	34	46	26	42	25

S = sucrose

K = KCl

DISCUSSION

The activity of isocitrate dehydrogenase from P.cinnamomi did not appear to be affected by growth at different potentials or on different osmotica. This result agrees with similar work on other microorganisms and higher plants. Johnson et al. (1968) found that the activity of glucose-6-phosphate dehydrogenase activity in Dunaliella viridis was the same whether grown in 1.28 molar NaCl (-6.2 MPa) or 3.75 molar (ca -22.5 MPa) when assayed at different concentrations of NaCl. Salt marsh plants showed markedly enhanced growth when cultured in 0.4 molar NaCl (-1.8 MPa) compared to dilute solutions but there was no difference in the specific activity of several enzymes nor were their pH optima altered (Greenway & Osmond, 1972). Arginase activity extracted from the diatom Cyclotella cryptica grown under normal conditions of 33% artificial sea water (-0.9 MPa) and temporarily high salt (80% ASW, -2.2 MPa) was similar either in the presence or absence of proline (Liu & Hellebust, 1976b). Lastly, alkaline phosphatase from the marine yeast Debaryomyces hansenii also showed no difference in such characteristics as molecular weight, Km, inhibitor constants, thermal half life or electrophoretic patterns whether grown in 2.7 molar NaCl (-14.6 MPa) or in dilute solution (Adler, 1978).

It should however be noted that these results were all obtained from experiments using saline culture media. The present work with P.cinnamomi appears to be the first indication that sucrose or KCl adjusted water potentials also do not alter enzyme characteristics. Calcium may act in a different way since changes in the calcium response of malate dehydrogenase extracted from Lemna minor grown in different concentrations of calcium were noted (Jefferies et al., 1969).

It must be concluded that, at least in the case of the one enzme examined here, that growth at low water potential does not depend on synthesis of isoenzymes but that adaptation must occur by alteration of the milieu within which the enzyme operates.

THE EFFECT OF WATER POTENTIAL AND SOLUTES ON ISOCITRATE DEHYDROGENASE ACTIVITY

Phytophthora cinnamomi was grown on 0 CYA, Penicillium chrysogenum on -2.5 MPa glucose and Chrysosporium fastidium on -10 MPa glucose in the usual way. Three to five grams fresh weight of colony were extracted in the appropriate buffer as described above. Each crude extract was assayed at a number of different potentials produced with some of the solutes which have been found to accumulate in the hyphae of these species.

RESULTS

The results are presented in Table 4.9, both in terms of the actual rates of NADP reduction and also as a percentage of the maximum observed rate for each species. Some of the results of both this and the previous experiment have been combined in Fig. 4.7. Effects due to both potential per se and to the specific solute were evident.

Hypoosmotic stress was apparent in all three species and as might have been expected was greatest in C.fastidium and least in P.cinnamomi. Maximum activity was observed in glycerol solutions in all three species and actual rates were about 50% greater in P.cinnamomi than in the other two species. The optimum potential for P.cinnamomi was -5 MPa glycerol which was below the limit for growth. The optimum potential was also below the optimum for growth in P.chrysogenum, but the two probably coincided in C.fastidium. Glycerol of course has been proposed as the principal osmoregulator or compatible solute in the last two species but was not detected in P.cinnamomi (Table 3.14) under these experimental conditions. Proline which was accumulated by the latter was equally as effective as glycerol in protecting isocitrate dehydrogenase activity in P.cinnamomi at -1.5 MPa, and slightly more effective than glycerol at

the lower potential of -2.5 MPa. In P.chrysogenum, proline allowed only half maximum activity at both potentials, and so was a less effective protective agent than glycerol. The effect of proline on C.fastidium was not tested.

All the sugars tested were to a greater or lesser degree inhibitory. Inhibition was considerably less in P.cinnamomi than in the other two species. Surprisingly, glucose was less inhibitory to P.cinnamomi than sucrose the sugar on which it had been grown, the relative values being 63% and 27% respectively at -2.5 MPa whereas the reverse was true for P.chrysogenum where activities were 5% and 27% respectively. The two sugars had the same effect on C.fastidium (12% and 11%). Likewise fructose at -10 MPa appeared to be no less inhibitory than glucose (7% and 9% respectively) which does not correspond with the stimulatory effect of this sugar noted in Chapter 3. They also inhibited P.chrysogenum similarly (30% and 38%) but to a lesser degree than C.fastidium. P.cinnamomi was inhibited least of all by -10 MPa glucose.

At comparable potentials, KCl was less inhibitory than sucrose for P.cinnamomi but slightly more so in P.chrysogenum and C.fastidium. Maximum growth for P.chrysogenum occurred at -2.5 MPa but its enzyme activity was reduced to a quarter of the maximum rate by -2.5 MPa KCl (Fig. 4.7). KCl was also more inhibitory than glucose for all three species. KCl at -10 MPa completely inhibited C.fastidium, although some activity was observed at higher potentials (max 10% at -1.5 MPa). Only low activity was observed in P.cinnamomi and P.chrysogenum at the lowest potential produced with KCl.

When glycerol as well as KCl was added to the buffer at a final potential of -10 MPa, there was some relief of inhibition in P.chrysogenum; the activity increased from 6% to 17%. In C.fastidium the improvement was minimal and the observed rate was only 4% maximum. However glycerol was able to dramatically increase the rate when added with glucose in both species. P.chrysogenum increased from 38% to 83% and C.fastidium from 9% to 74% at -10 MPa. Thus glycerol has been demonstrated to act as a compatible solute, at least in the case of sugars, if not of ions. It may be that catabolite repression of

respiration (Table 4.7) has been modified by glycerol as Brown (1975) suggested occurred with arabitol in Saccharomyces rouxii.

Preliminary results did not suggest any relief of inhibition by KCl or glucose when the substrate concentration was increased ten fold.

NAD specific isocitrate dehydrogenase activity was not detected, although it is known to be present in addition to the NADP specific enzyme in Aspergillus niger (Ramakrishnan & Martin, 1955), Neurospora crassa (Sanwal et al., 1964) and Saccharomyces cerevisiae (Bernofsky & Utter, 1966).

Table 4.9. The effect of different solutes and osmotic potentials on NADP isocitrate activity from Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium.

ψ_s (-MPa)	Enzyme activity					
	P.cinnamomi		P.chrysogenum		C.fastidium	
	rate*	%max	rate*	%max	rate*	%max
0 (sucrose)	4.29	67	2.14	55	0.47	13
1.5 (sucrose)	2.79	43	1.57	40	0.45	13
1.5 (KCl)	3.86	60	1.24	32	0.34	10
1.5 (glycerol)	4.72	73	3.30	85	1.68	47
1.5 (proline)	4.50	70	1.98	51	-	-
2.5 (sucrose)	1.71	27	1.37	35	0.40	11
2.5 (glucose)	4.07	63	1.90	49	0.42	12
2.5 (KCl)	3.00	47	0.91	23	0.18	5
2.5 (glycerol)	4.07	63	3.46	89	2.00	56
2.5 (proline)	4.72	73	1.98	51	-	-
5.0 (glucose)	3.86	60	1.81	47	0.37	10
5.0 (KCl)	1.29	20	0.50	13	0.05	1
5.0 (glycerol)	6.43	100	3.87	100	2.73	77
10.0 (glucose)	2.57	40	1.49	38	0.32	9
10.0 (KCl)	0.43	7	0.25	6	0	0
10.0 (glycerol)	3.86	60	3.87	100	3.57	100
10.0 (fructose)	-	-	1.15	30	0.24	7
10.0 ($\frac{1}{2}$KCl + $\frac{1}{2}$glycerol)	-	-	0.66	17	0.13	4
10.0 ($\frac{1}{2}$glucose + $\frac{1}{2}$ glycerol)	-	-	3.22	83	2.63	74
20.0 glycerol	-	-	-	-	2.73	77
4 millimolar isocitrate						
10.0 (glucose)	-	-	0.16	4	0.26	7
10.0 (KCl)	-	-	-	-	0	0

* µmole NADP reduced/min/mg protein x 10^2

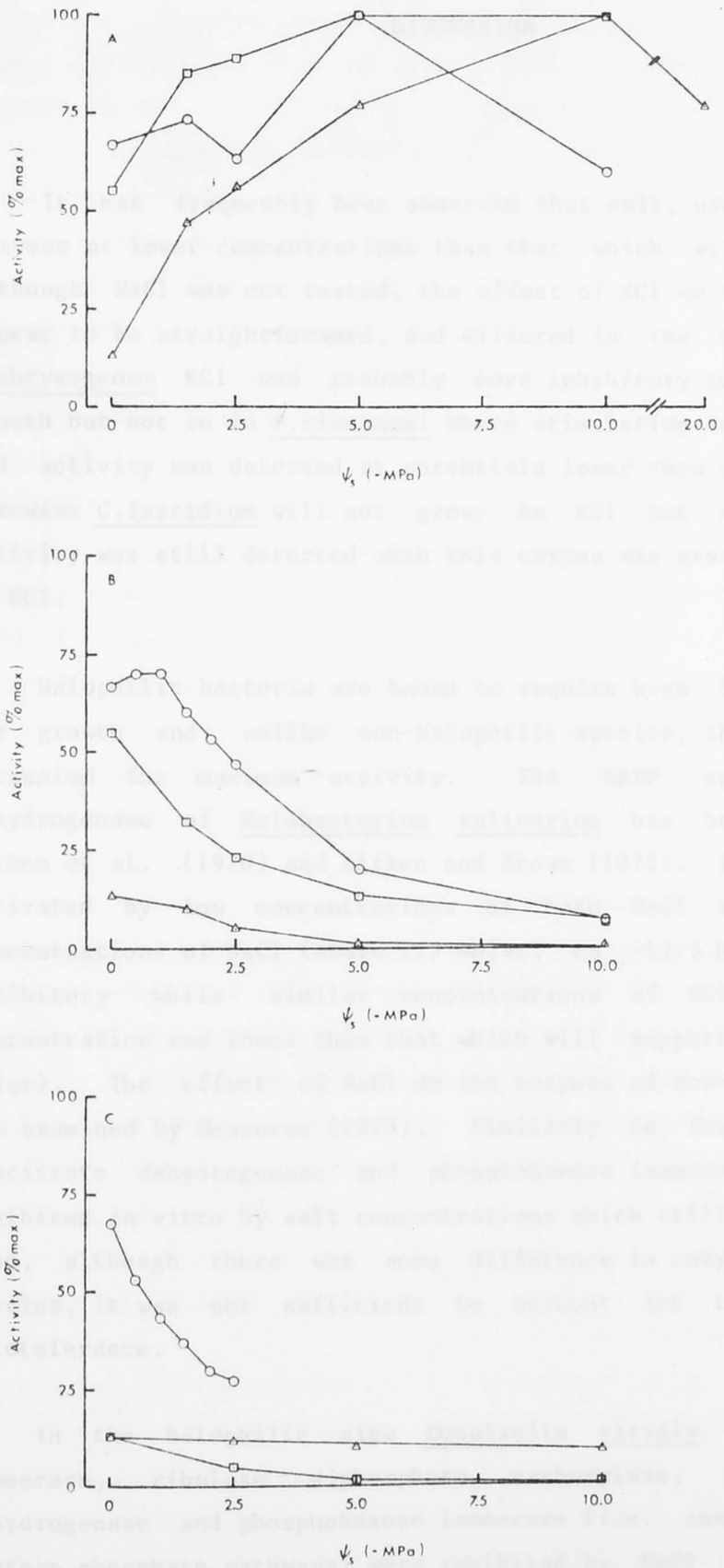


Fig. 4.7. Effect of water potential on NADP isocitrate dehydrogenase activity from Phytophthora cinnamomi (○), Penicillium chrysogenum (□) and Chrysosporium fastidium (△) assayed with glycerol (A), KCl (B) and sucrose (C) or glucose (△,□) (C).

DISCUSSION

It has frequently been observed that salt, usually NaCl, inhibits enzymes at lower concentrations than that which will support growth. Although NaCl was not tested, the effect of KCl on these fungi does not appear to be straightforward, and differed in the three species. In P.chrysogenum KCl was probably more inhibitory to the enzyme than to growth but not so in P.cinnamomi where stimulation occurred at -0.5 MPa and activity was detected at potentials lower than will support growth. Likewise C.fastidium will not grow on KCl but some slight enzyme activity was still detected when this enzyme was assayed in the presence of KCl.

Halophilic bacteria are known to require high NaCl concentrations for growth and, unlike non-halophilic species, their enzymes require potassium for maximum activity. The NADP specific isocitrate dehydrogenase of Halobacterium salinarium has been characterised by Aitken et al. (1970) and Aitken and Brown (1972). It was found to be activated by low concentrations of both NaCl and KCl, but high concentrations of NaCl (above 2.5 molar, ca -13.3 MPa) were severely inhibitory while similar concentrations of KCl were not. This concentration was lower than that which will support growth (3 to 5 molar). The effect of NaCl on the enzymes of non-halophilic bacteria was examined by Measures (1975). Similarly he found that aldolase, isocitrate dehydrogenase and phosphohexose isomerase were all totally inhibited in vitro by salt concentrations which still permitted growth. Also, although there was some difference in enzyme activity between species, it was not sufficient to account for the differences in halotolerance.

In the halophilic alga Dunaliella viridis, pentose phosphate isomerase, ribulose diphosphate carboxylase, glucose-6-phosphate dehydrogenase and phosphohexose isomerase (i.e. enzymes of the EMP and pentose phosphate pathways) were inhibited by NaCl concentrations far lower than that in the growth medium (3.75 molar or about -22.5 MPa) (Johnson et al., 1968). KCl and other monovalent cations were also

inhibitory. However growth of whole cells was optimum at 0.8 to 2 molar NaCl (-3.7 to -10.1 MPa) and in this case the effect was specific to Na^+. Borowitzka & Brown (1974) tried to explain the halophily of this genus in terms of isoenzyme synthesis such as occurs in the halophilic bacteria, but none was detected. The alternative explanation suggested was that salt was excluded from the cells. However, the sodium content was difficult to measure in these organisms and they found only indirect evidence that Na^+ was excluded although there was a moderate uptake of K^+. Later work has in fact shown that D.parva does contain significant amounts of Na^+ (Gimmler & Schirling, 1978). Borowitzka and Brown also demonstrated that glycerol accumulated by Dunaliella did not overcome the inhibition by salt of glucose-6-phosphate dehydrogenase, although the added glycerol in these experiments would also have lowered the potential which might explain this result. Glycerol alone did not inhibit this enzyme from either species until a concentration of about 3 molar was reached (-9.8 MPa). There was no difference in the responses of the enzymes of D.viridis and D.tertiolecta so that their different tolerance to salt in the growth medium could not be explained on this basis.

In contrast the alkaline phosphatase from the marine yeast Debaryomyces hansenii, but not that from Saccharomyces cerevisiae, was broadly stimulated by KCl up to concentrations of several molar (Adler, 1978). The stimulation appeared to be the result of alteration of the activity of the enzyme (Vmax) rather than its affinity for the substrate (Km). Added glycerol reduced the activity by altering both parameters, but only slightly. When glycerol and salts were combined in the assay solution, the results were intermediate. Adler suggested that the competitive effect beteen glycerol and water might interfere with the transfer of the phosphate group.

Among the higher plants, the halophytes also do not appear to contain enzymes noticeably different from other species. Malate dehydrogenase, aspartate transaminase, glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase from halophytes were assayed at NaCl concentrations of up to 0.5 molar (-2.3 MPa), a concentration well below that in the cell sap and all were to some degree inhibited; KCl gave very similar results (Greenway & Osmond, 1972). However this was a salt

effect and not due to water potential since isoosmotic mannitol showed little inhibition. Likewise Triglochin maritima was inhibited by NaCl but not by proline (Stewart & Lee, 1974).

To summarise, the response of isocitrate dehydrogenase from P.chrysogenum to KCl appears to be similar to the non-halophilic bacteria, Dunaliella and the higher plant halophytes. Three different compatible solutes have been postulated; three; proline in P.cinnamomi, K^+ in the halophilic bacteria and glycerol in D.hansenii. The enzyme activity of C.fastidium was clearly very low in the presence of KCl, possibly explaining the inability of this species to tolerate salt in the growth medium.

Clearly generalisations cannot be made from results obtained with a single enzyme, and the response of the whole pathway must be examined to appreciate the overall impact of inhibitors. The effect of chloride salts on the glycolysis sequence in extracts from pea seeds was examined by Greenway et al. (1977) for this purpose. The inhibitory effect was found to act on steps after the formation of fructose-1-6-diphosphate, so that there was less control by the regulatory enzyme phosphofructokinase. The enzymes most affected were glyceraldehyde-3-phosphate dehydrogenase and aldolase (Greenway & Setter, 1977).

Substrate concentration effects were mentioned in the introduction as a possible explanation for the phenomenon of salt inhibition. More detailed studies on the effects of KCl on malate dehydrogenase from T.maritima were carried out by Greenway and Sims (1974) to examine this possibility. They found that the degree of inhibition was largely a function of the concentration of the substrate and so suggested that KCl reduced affinity of the enzyme for both substrate and some allosteric inhibitors. It is obviously important that if the substrate level is to increase to overcome this type of inhibiton, that it does not affect the rates of other reactions in the sequence. There was some evidence that this response occurred in peas (Greenway & Setter, 1977) and in some microorganisms, although not all the inhibition by salt was overcome by increase in substrate concentrations. There was no evidence in these

preliminary experiments (Table 4.9) that an increase in substrate level diminished inhibition of fungal isocitrate dehydrogenase.

The theory of compartmentalisation which has been advanced also by a number of workers, suggests that toxic ions are spatially separated from salt sensitive enzymes which instead are protected by a compatible solute, usually an organic compound, although the potassium ion is also able to function in this way in halophilic bacteria. This solute must be in water potential equilibrium with the salt containing compartment. This theory is most plausible in the higher plants where the large vacuole and the cytoplasm constitute natural compartments. There is some evidence for its occurrence in Suaeda maritima; specific staining suggested that glycine betaine accumulated for osmoregulation was largely restricted to the cytoplasm (Hall et al., 1978). However Johnson et al. (1968) thought this unlikely to occur in the alga D.viridis since supersaturating conditions would result in the part of the cell containing the salt. It also seems improbable that such a system should operate in fungi where there are no significant vacuoles in the growing hyphal tip. However there is some recent evidence that compartmentalisation may occur in yeast. Hüber-Walchli and Wiemken (1979) were able to separate vacuoles from cytoplasm in Candida utilis cells. Glutamate was the only free amino acid to be found predominantly in the non-vacuolar fraction, as were K^+, Na^+, orthophosphate and ATP. Roomans and Sevéus (1976) also considered that concentrations of ions in the cytoplasm and nucleus of S.cerevisiae were similar and about twice that in the vacuole. However the cells were not grown under osmotic stress so that there is no information on distribution of osmoregulatory compounds.

Few of the enzymes mentioned so far have also been assayed at low water potentials produced with sugars. Mitochondrial NADH oxidase from yeast has been examined by Brown (1975). The same result was obtained as for oxygen uptake experiments; that is when grown in a basal medium the enzyme from S.cerevisiae was slightly more active than that from S.rouxii, but was more inhibited by low water potential whether from added PEG or glucose. This is analagous to the results obtained in the present experiment in that expected responses in terms of hypoosmotic and hyperosmotic stress

were obtained when glycerol alone was used, but did not apply to glucose which in fact was least inhibitory to the non-osmophilic species in the absence of compatible solutes. The inhibition by glucose seems to be a general phenomenon which, like inhibition by polyols, may be due to their effect on the dielectric constant although the inhibition by sucrose cannot be explained on this basis (Brown, 1978). He found that fructose was the least inhibitory of the sugars tested of activity of isocitrate dehydrogenase from yeast. The ability of glycerol to alleviate repression by glucose has clearly been demonstrated. It would be of interest to discover whether it is also effective against sucrose inhibition, and whether proline can fulfil the same function for enzymes of P.cinnamomi.

The mechanism of action of compatible solutes has to some extent been explained by various workers. Myers and Jakoby (1973) examined the effect of glycerol and other polyhydric compounds on sixteen enzymes, although these did not include isocitrate dehydrogenase. Assays with and without 30% (v/v) glycerol (-15.9 MPa) were compared and both the affinity for substrate (Km) and maximum rate (Vmax) were found to be altered by glycerol. The latter decreased in all but two of the enzymes, while Km was either unaltered or reduced. This was interpreted as classical inhibition by glycerol. Glycine betaine, glycerol, proline and sucrose were much less inhibitory at 0.5 molar (ca -1.3 MPa) to malate dehydrogenase from barley than similar potentials of NaCl or KCl (Pollard & Wyn Jones, 1979). The protective effect of glycine betaine was thought to result from NaCl-glycine betaine-protein-water interactions and not to binding of glycine betaine and protein. Proline and glycerol proably afford protection to enzymes by the latter mechanism.

Schobert (1977) and Schobert and Tschesche (1978) have proposed that proline has special properties such that it enhances the solubility of proteins. This it does by changing hydrophobic groups to hydrophilic ones, so releasing more water into solution as well as stabilising the protein. Glycerol is proposed to replace water molecules because of its OH group and so participate in the hydrophilic side chains with the same result. Thus under conditions of reduced water content, the hydration and hence activity of enzymes would be maintained.

Bull and Breese (1978) compared binding of some alcohols and polyhydric alcohols, including glycerol, to several proteins. Alcohol binding was probably accompanied by dehydration while polyol binding was not. Interestingly, sucrose produced the greatest degree of hydration, and glycerol and ethylene glycol the least. Sucrose was not bound to the protein but the degree of glycerol binding was difficult to determine. Comparable experiments on the binding of salts by proteins show that the effect is specific to the particular salt. Only sodium sulphate did not reduce the degree of hydration (Bull & Breese, 1970). Whatever the solutes bound to the protein, the water of hydration must be in thermodynamic equilibrium with the bulk solution. If the potential of the latter is lowered by the presence of solutes then the water of hydration must also decrease as dictated by the water sorption isotherm of the protein (Griffin, 1981).

Finally the enzymes actually involved in the synthesis and degradation of compatible solutes themselves have been examined in some cases, although not in filamentous fungi. In the alga Cyclotella cryptica which accumulates proline in response to osmotic stress, the effect of proline on two enzymes involved in proline synthesis were examined (Liu & Hellebust, 1976b). Proline had no effect on the activity of either arginase or pyrroline-5-carboxylate reductase. Proline oxidase from spinach was not inhibited by added sucrose, mannitol or polyethylene glycol of potentials of -2 MPa (Huang & Cavalieri, 1979). A stimulatory effect of K^+ on glutamate dehydrogenase in some non-halophilic bacteria was noted by Measures (1975). The α-glycerophosphate oxidase concentration of Candida utilis increased when the yeast was grown on glycerol, and also the α-glycerophosphatase activity in S.cerevisiae was dependent on a low concentration of ions (Gancedo et al., 1968). Borowitzka and Brown (1974) found that glycerol enhanced the activity of glycerol dehydrogenase up to about 6 molar (ca -27.8 MPa). Further work is needed on the effect of both potential and osmoticum type on the enzymes responsible for synthesis and regulation of compatible solutes in filamentous fungi.

5. THE EFFECT OF WATER POTENTIAL ON HYPHAL GROWTH, MORPHOLOGY AND ULTRASTRUCTURE

INTRODUCTION

The importance of morphology in the identification of fungi is axiomatic, but the profound effect which conditions of growth can have on these characteristics is not always appreciated. The dimorphism of Mucor is an extreme example. The inhibition of sporulation and the transpiration of considerable amounts of fluid by Penicillium chrysogenum at high water potentials have been striking examples in the current work. Although prescribed media are now generally used for taxonomic work, the water potential of the medium is not usually considered. Robertson (1959) was the first to recognise the importance of the connection between water potential and growth, and initiated work in this direction. He certainly succeeded in establishing the complexity of the relationship, if not in unravelling it.

Since one of the most obvious effects of low water potential is on the growth rate, it may prove that water potential can be used as a tool to investigate the mechanism of growth. The morphology and ultrastructure of the same three species of fungi have thus been examined with the light and electron microscope to assess the effects of water potential, both steady state and shock.

MATERIALS AND METHODS

Fungi and media

Media were prepared as before unless mentioned otherwise. The same three species were used for the experiments.

Light microscopy

Squares were cut in the cellophane and lifted, with a portion of the colony margin onto a clean glass slide. The preparation was mounted in 2% cotton blue in lactophenol, and examined immediately. This mountant caused what was assumed to be drastic plasmolysis in C.fastidium (Plate. 5.10) and so the hyphae of this species were mounted unstained in glucose solutions of the same water potential as the hyphae.

Hyphae were examined with bright field or phase contrast optics on a Wild M 20 microscope at magnifications of 100x, 200x and 400x. Measurements were made using an eyepiece micrometer calibrated with a stage micrometer.

In the shock experiments, hyphae were examined unmounted, directly on the cellophane overlying the agar, employing phase optics. Millipore 47 mm diameter Petrislides with lids containing ca 3 ml agar were used for the purpose. Photographs were taken with a Nikon Polaroid camera. Growth measurements were made from the photographs which included a lens micrometer calibrated against a stage micrometer.

Transmission electron microscopy

Phosphate buffer (pH 5.8) was adjusted to the same water potential as the hyphae with the same osmoticum as that used in the growth medium. The water potentials of the solutions were checked psychrometrically, and a further check was made, using the light microscope, to ensure that bursting of tips did not occur. Fixative of 3% glutaraldehyde made up in the appropriate buffer was flooded directly onto the colony in the

petri dish, and in some cases, molten agar (45°) of the same potential was poured on top, to attempt to prevent the hyphal tips curling back. This procedure was followed only in the case of P.cinnamomi since the agar did not adhere to the hyphae in the other two species. When the agar had set, rectangles of agar containing pieces of colony margin were post-fixed in 2% osmium tetroxide, also made up in buffer of the appropriate potential, for 2 h. Material was washed in four changes of distilled water for 1 h and then dehydrated through a series of alcohol and water mixtures. It was then rinsed twice in propylene oxide, passed through a series of propylene oxide and Spurr's resin mixtures and finally embedded in Spurr's resin. Blocks were polymerised overnight at 70°C.

Thin sections were cut from the blocks using diamond knives and a Reichert ultramicrotome, mounted on copper grids and post-stained with aqueous uranyl acetate and lead citrate. The sections were examined in a Hitachi HU 12 and subsequently a Jeol 100C electron microscope. Measurements of wall thickness were made from electron micrographs, using a Bausch and Lomb 7x micrometer lens.

THE EFFECT OF STEADY STATE OSMOTIC STRESS ON HYPHAL ULTRASTRUCTURE

Each of the three species was grown at four different water potentials; Phytophthora cinnamomi at 0, -1, -2 and -3 MPa sucrose, Penicillium chrysogenum at 0, -2.5, -5 and -10 MPa glucose and Chrysosporium fastidium at -5, -10, -15 and -20 MPa glucose. Fifty two mm diameter plastic Petri plates were overlain with cellophane and inoculated centrally on different days so that all the colonies were about 10 mm radius at the time of sampling. Material for electron microscopy was prepared as described above.

Two series of sections were cut from the blocks; one to obtain transverse sections of hyphae so that the thickness of the wall could be determined, and in the other, an attempt was made to obtain longitudinal sections through the hyphal tip so that any effect of potential on the organisation of the apex could be seen.

RESULTS

Transverse sections

The effect of water potential on wall thickness was significantly greater than the hyphal variation in all three species, but the trend differed in P.cinnamomi from that in the other two species (Table 5.1). Not only were the walls of P.cinnamomi considerably thicker than in P.chrysogenum or C.fastidium, but the thickness increased as the potential decreased; the wall was more than three time thicker at -3 MPa than it was at -1 MPa. Hypoosmotic stress however caused a slight thickening of the walls of P.chrysogenum and C.fastidium. Walls from adjacent potentials did not usually differ significantly (P=0.05) in thickness, except in the cases marked. It has been assumed for this analysis that plasmolysis did not occur and that the wall is equivalent

to that part of the cell surrounding the cytoplasm.

Table 5.1. The effect of potential on wall thickness in Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium. Differences in wall thickness in each species were tested by analyses of variance and by least significant differences. LSD (P=0.05) were 123 nm, 9 nm and 9 nm for the three species respectively. Values from adjacent potentials which differ significantly are marked with a bar.

P.cinnamomi*		P.chrysogenum**		C.fastidium**	
ψ_s (-MPa)	wall thickness (nm)	ψ_s (-MPa)	wall thickness (nm)	ψ_s (-MPa)	wall thickness (nm)
0	172 ± 44	0	75 ± 15	5	60 ± 18
1	131 ± 46	2.5	80 ± 16	10	43 ± 12
2	318 ± 185	5	58 ± 3	15	46 ± 14
3	425 ± 196	10	63 ± 18	20	40 ± 13

* N = 10

** N = 20

The variation in cell size was also examined by comparing the diameters of the transverse sections. The differences between potential were greater than the variability among hyphae only in C.fastidium, and the only cells from adjacent potentials significantly different in size were the -5 and -10 MPa treatments (Table 5.2). This appeared to differ from the results obtained from the light microscope, where the diameter of the apex, and the hypha 100 µm behind the apex, from -5 MPa were both less than the values obtained for the other potentials in C.fastidium

(Table 5.7).

The cell size of P.cinnamomi did not increase as the potential decreased so that the wall occupied a greater proportion of the whole cell at low potentials. Likewise the cell size did not change in P.chrysogenum so that the wall occupied a slightly greater proportion at 0 and -2.5 MPa than at lower potentials. However in C.fastidium the cell diameter as well as the wall thickness increased at -5 MPa so that an approximately constant proportion of wall to cell was maintained.

Table 5.2. The effect of potential on cell size (diameter) in Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium. Differences in cell diameter in each species were compared by analyses of variance and least significant differences. LSD (P=0.05) for C.fastidium was 0.40 μm. Values from adjacent potentials which differed significantly are marked with a bar.

P.cinnamomi*		P.chrysogenum**		C.fastidium**	
ψ_s (-MPa)	Cell diameter (μm)	ψ_s (-MPa)	Cell diameter (μm)	ψ_s (-MPa)	Cell diameter (μm)
0	4.16 ± 1.20	0	2.91 ± 0.45	5	3.01 ± 0.89
1	4.67 ± 0.92	2.5	3.28 ± 0.55	10	2.46 ± 0.54
2	4.18 ± 1.12	5	3.08 ± 0.42	15	2.30 ± 0.45
3	3.80 ± 0.49	10	3.14 ± 0.61	20	2.09 ± 0.55

* N = 10

** N = 20

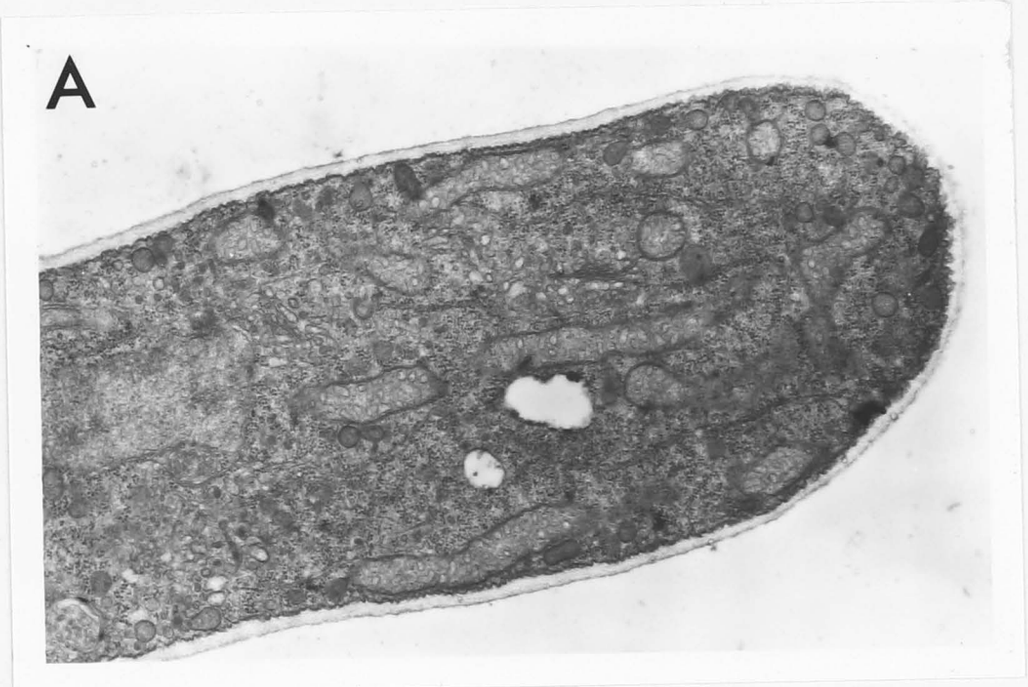
Longitudinal sections

The sectioning of hyphal tips in order to obtain median sections through the apex proved difficult and time consuming and was only partially successful. A thorough analysis would have required a much larger and more intensive study.

The criterion for judging whether or not the apex had been sectioned was the presence of numerous wall vesicles (Beckett et al., 1974). Sections from each of the P.cinnamomi treatments are shown in Plates 5.1 and 5.2. The first three figures are possible tip sections as judged by the vesicle criterion, although the fact that mitochondria are also evident close to the tip makes that assumption more doubtful. Plate 5.2B is clearly not a tip section, but it does serve to illustrate the wall structure. Microfibrils can be seen in the middle section of the wall and what is presumably membraneous material in the otherwise unstained inner layer. The progressive thickening of the wall as the potential decreased, which has already been noted in the transverse sections, can be seen from the four longitudinal sections also. Below 0 MPa sucrose, the wall appears to consist of three layers.

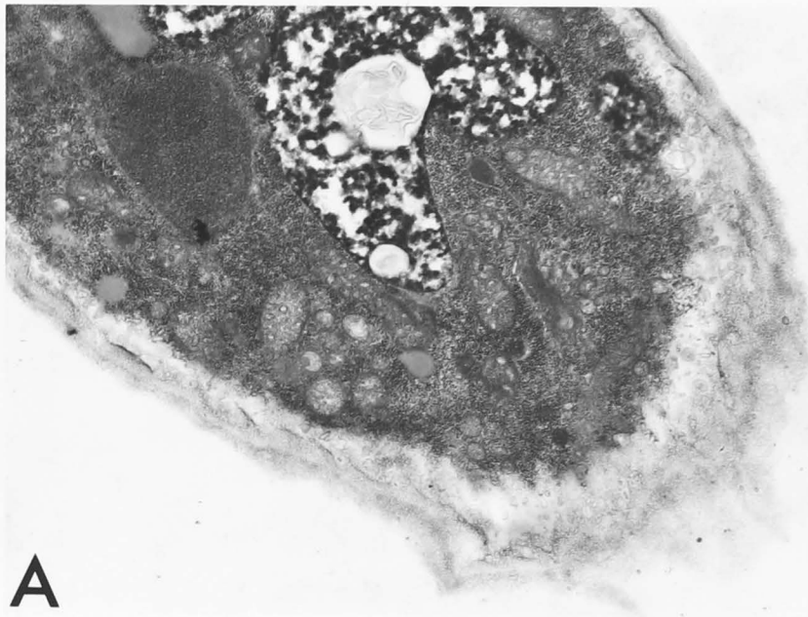
Unfortunately the quality of the sections is not sufficiently good to draw any conclusions concerning the effect of potential on the organisation of the apex such as the number or size of vesicles. Only one possible tip section was obtained from the P.chrysogenum series (Plate 5.3) and none at all from C.fastidium. Collinge et al. (1978) have also studied the ultrastructure of P.chrysogenum grown at a high water potential, and there were no obvious differences between the tip structure they observed and that shown here at -2.5 MPa KCl. It is not possible to detect clearly the Spitzenkörper common to higher fungi in this section (Grove & Bracker, 1970).

Plate 5.1. Possible median sections through the apex of Phytophthora cinnamomi grown on 0 MPa (A) and -10 MPa sucrose (B). Apical vesicles of ca 0.15 µm with granular contents can be seen. Note thickening of the wall. Magnification x 20,800.


A


B

Plate 5.2. Possible median sections through the apex of Phytophthora cinnamomi grown on -2 MPa sucrose (A) and -3 MPa sucrose (B). Apical vesicles can be seen in A. There is a thin outer layer, microfibrils appear in the middle layer of the wall in B and amorphous membraneous material in the inner layer. Magnification x 16,900.


A


B

Plate 5.3. Possible median section through the apex of Penicillium chrysogenum grown on -2.5 MPa KCl. Apical vesicles of ca 0.04 μm can be seen but not microvesicles. Note the absence of ribosomes (electron dense granules) in the first 0.5 μm, and the thinner wall of the apex. Magnification x 50,000.

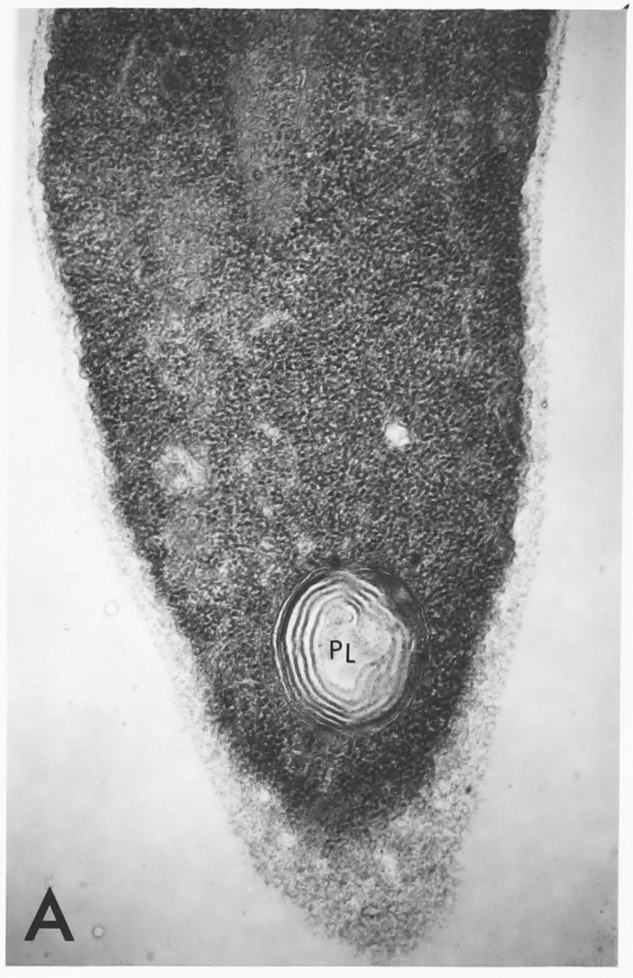

0.5µm

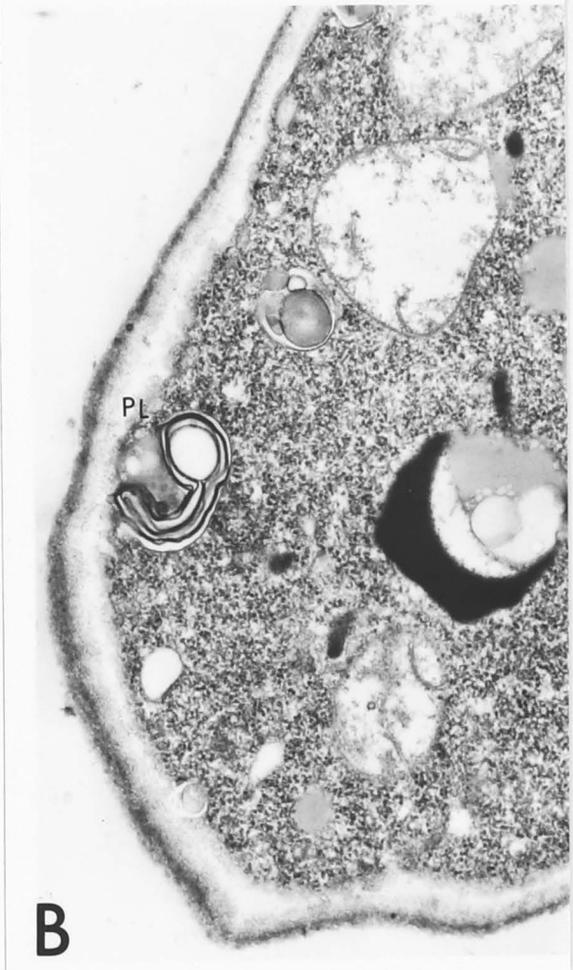
The sections did reveal a number of striking membraneous structures (Plates 5.4 & 5.5). The membrane whorl, myelin figure or plasmalemmasome shown in Plate 5.4A has also been reported in P.chrysogenum by Trinci & Righelato (1970) although they found them usually to occur within larger vacuoles. They were unable to suggest any function for this structure but Beckett et al. (1974) reported their presence to be associated with conidiogenesis in Ascomycetes. It appears that the membrane structure degrades to become large vesicles containing membrane fragments which probably contribute to wall formation. Marchant and Moore (1973) have demonstrated that they are not artifacts of fixation as was previously thought, but have a variable structure consisting of vesicles, tubules or arrays of membrane lamellae. A somewhat similar structure occurred in a section of C.fastidium grown at -5 MPa (Plate 5.4B). The proximity of the 'tail' of this structure to the wall might suggest some functional connection; other vesicles or lomasomes apparently coalescing with the wall can be seen also in this section.

Numerous folded membranous structures were also seen in C.fastidium (Plate 5.5A) often at the boundaries of the cytoplasm, and their detailed structure is shown in Plate 5.5B. These structures bear some resemblance to the mesosomes or infoldings of the cell membrane which are found in gram-positive bacteria. Despite considerable effort, little is known of the structure or function of mesosomes (Salton, 1978). They lack the enzymes which are commonly found on the bacterial plasma membrane, but have some antigenic activity and it is these features which have led to the suggestion that "they may represent the peeling off or rearrangement of the outer leaflet of the plasma membrane due to perturbation of the latter structure". However Rogers et al. (1978) consider that they are not directly involved in wall formation, but they may mark the position of future septa.

Typical lomasomes were found in P.cinnamomi (Plate 5.4C). These are probably the result of membrane fusion which occurs with the expulsion of the contents of the apical vesicles in the extracellular space (Girbardt, 1969). Marchant and Moore (1973) considered that they were waste material which has been accumulated and trapped by the cell wall.

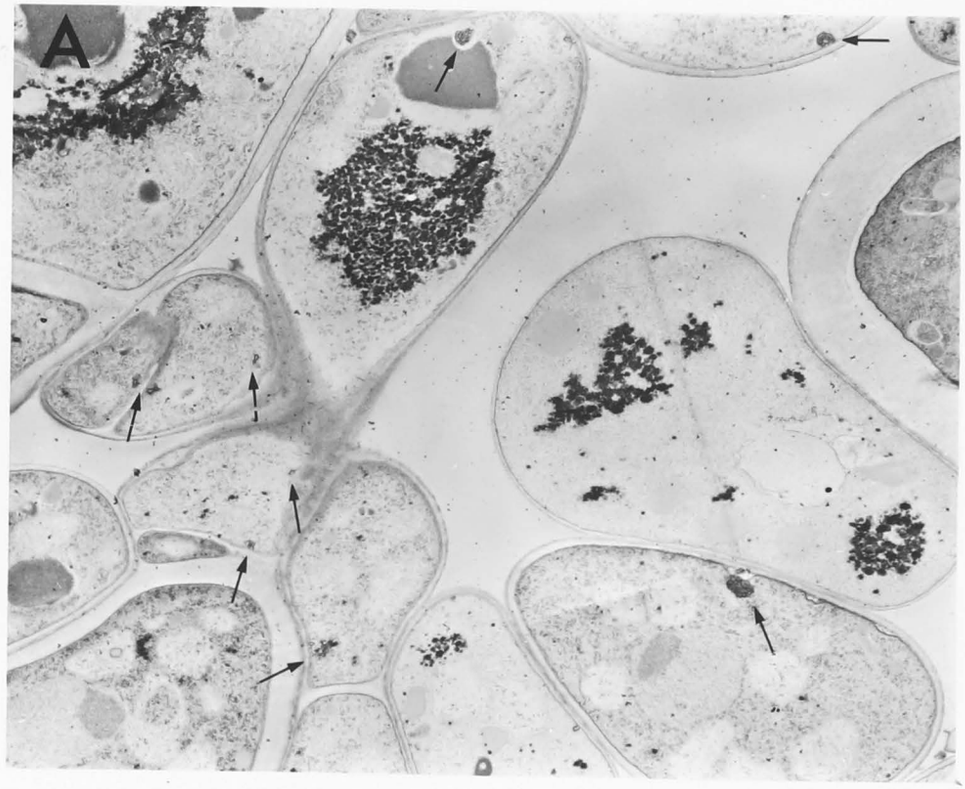
Plate 5.4. Plasmalemmasomes and lomasomes. Plasmalemmasomes (PL) in Penicillium chrysogenum on -2.5 MPa glucose x 26,000 (A), and Chrysosporium fastidium on -5 MPa glucose x 33,800 (B) and lomasomes (L) extending both into and away from the cytoplasm of Phytophthora cinnamomi grown on -2 MPa sucrose x 20,800 (C).

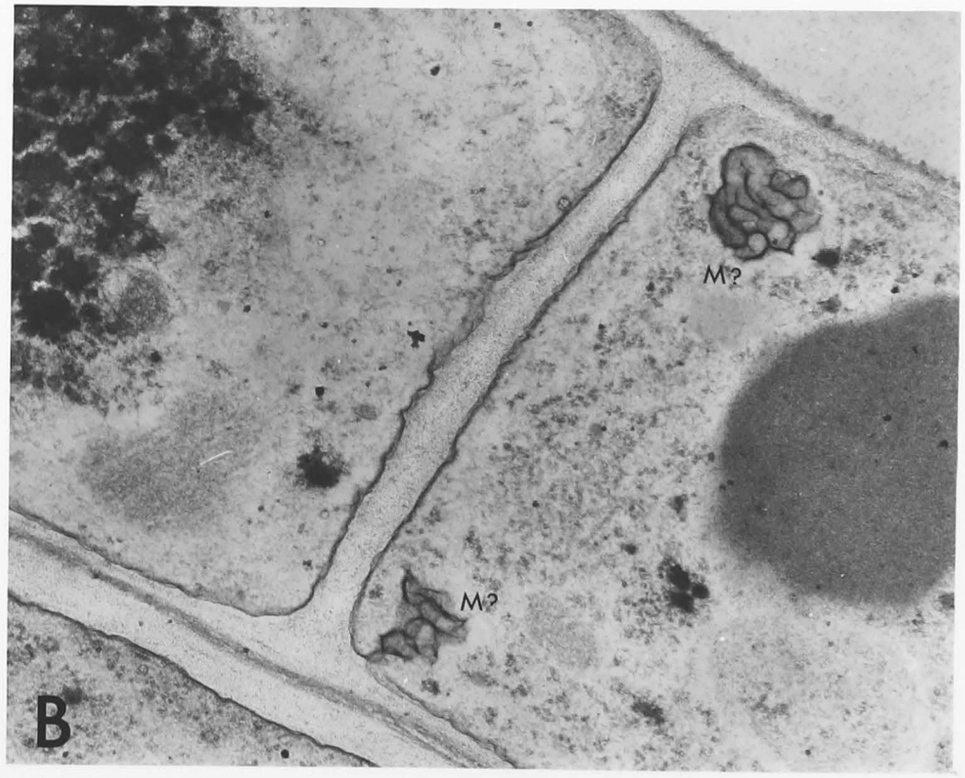

PL
A


PL
B


C
L
L
L

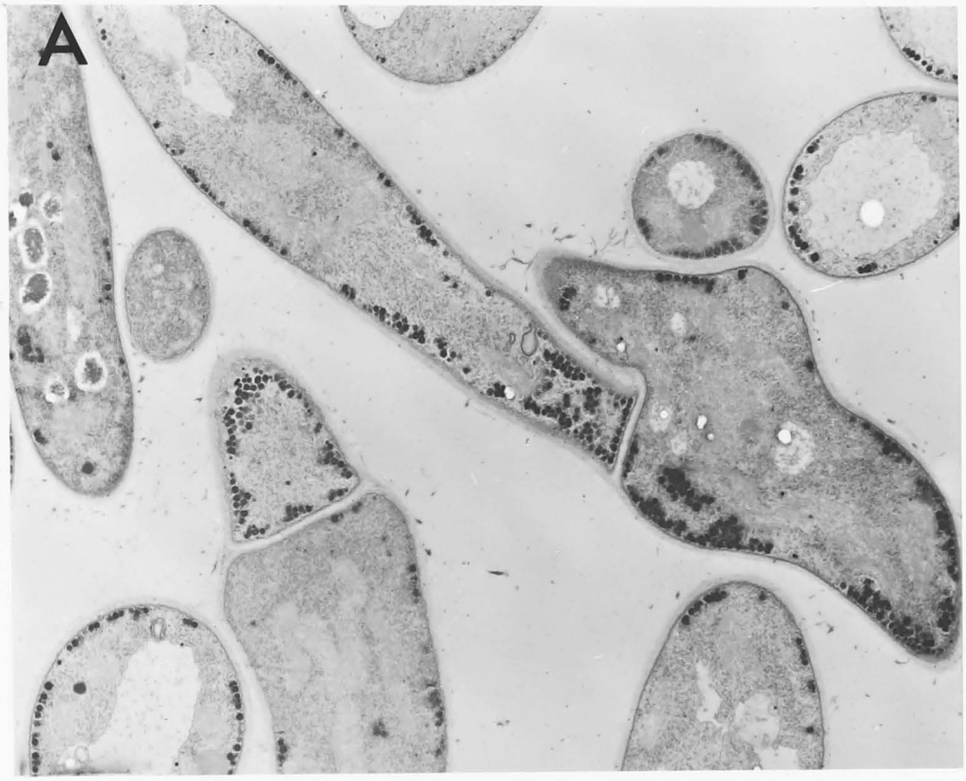
Plate 5.5. Mesosomes? Folded membrane structures observed in Chrysosporium fastidium on -5 MPa glucose x 7,600 (arrows) (A) and x 38,200 (M?) (B).


A


M?
M?
B

Glycogen granules were evident when the material was prepared in buffer of pH 7.0 but not pH 5.6. The evidence for assuming that these structures are glycogen is the dimensions of the α-glycogen particles (ca 25 nm) of which the β-glycogen rosettes were composed (Plate 5.8) (Beckett et al., 1974), and that they were not stained at low pH and in fact 'holes' appear where the glycogen probably had been when sections were post-stained with uranyl acetate in addition to lead citrate (Plate 5.6B) (A. Pyliotis, pers. comm.). These probably correspond to the "diffuse, electron transparent areas present in the cytoplasm adjacent to the hyphal wall ---- never bounded by a membrane" seen by Righelato et al. (1968) in P.chrysogenum. The granules appeared to be arranged around the margin of P.chrysogenum cells (Plate 5.6A), but in clusters more centrally in C.fastidium (Plate 5.7A). Granules were not evident in P.cinnamomi or the thick walled spores of C.fastidium (Plate 5.7). Interestingly, virus particles have also been found in several strains of P.chrysogenum (Banks et al., 1969). However these are polyhedral and 35 nm in diameter and so larger than α-glycogen particles (15-30 nm).

Plate 5.6. Glycogen granules around the margins of Penicillium chrysogenum cells (0 CYA) (A) but absent leaving 'holes' after staining with uranyl acetate (-2.5 MPa glucose) (B). Magnification x 8,000.


A

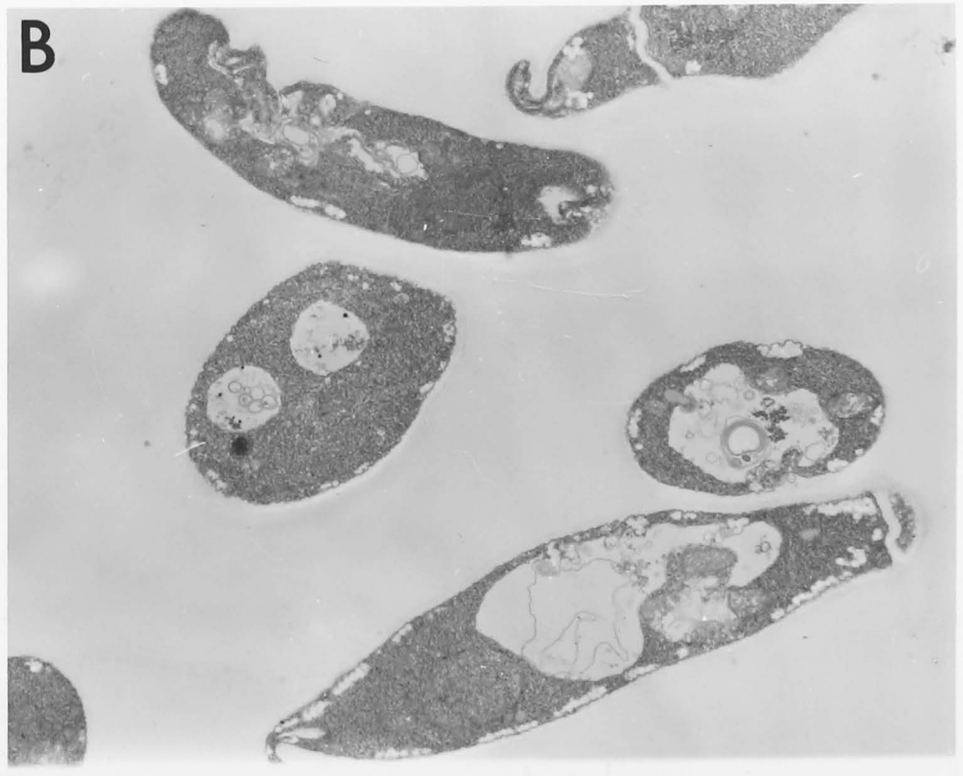
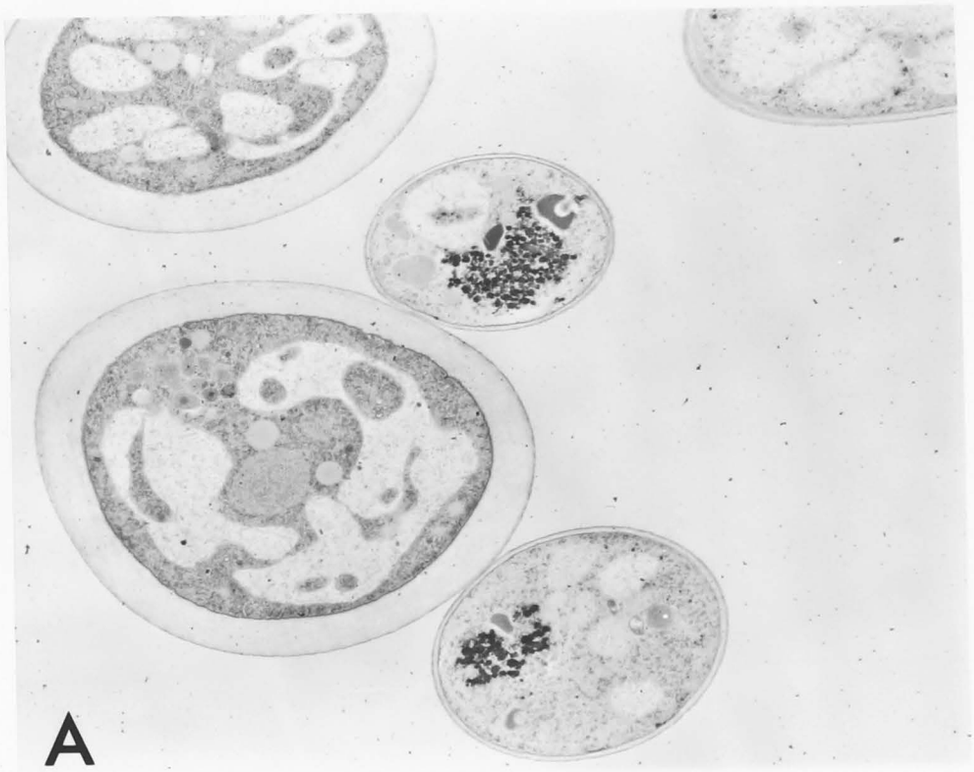

B

Plate 5.7. Glycogen. Chrysosporium fastidium on -5 MPa glucose (A) also contained glycogen in central clusters in the thin walled hyphae but not in the thick walled spores. Glycogen was not observed in Phytophthora cinnamomi on 0 CYA (B). Magnification x 8,000.


A


B

Plate 5.8. Detail of β-glycogen rosettes showing 25 nm α particles in a section of Penicillium chrysogenum grown on 0 CYA. Magnification x 60,000.


0.5 µm

DISCUSSION

The ultrastructure of P.chrysogenum is well known and the structure of the walls of Phytophthora has also been investigated but there is no information on C.fastidium in this respect. Righelato et al. (1968) considered the effect of growth rate on ultrastructure of P.chrysogenum. They used a chemostat and varied the growth rate by changing the glucose supply rate, but found little difference in mean wall thickness although the minimum value was 62 nm and the maximum 280 nm. Thus liquid culture appeared to result in somewhat thicker walls than young colonial growth (Table 5.1). The growth rates obtained were all considerably lower than those obtained in the experiments reported here, and so were probably all under hypoosmotic stress. Similarily, Collinge et al. (1978) found that the walls of the same species grown at pH 8 were thinner than those grown at lower pH, and also when carbon was limiting in the growth medium the walls were thinner than when nitrogen was limiting. Thus cultural conditions are an important determinant of wall thickness.

The wall of the extension zone (1.5 μm long in P.chrysogenum) and of young apex of P.cinnamomi have been shown to be thinner than the mature hyphal wall, and this was also demonstrated in P.chrysogenum at -2.5 MPa KCl (Plate 5.3). The thickness of the wall of P.cinnamomi from 0 MPa CYA (172 nm) was very similar to that of Phytophthora parasitica grown on a defined medium of high water potential (176 nm) (Hunsley, 1973), but the excessive wall thickening observed at lower potentials must be distinguished as 'unbalanced' wall growth as defined by Trinci (1978), although this condition has usually been observed to occur either in the centre of old colonies or when nutrient is limiting. It is well known that the walls of the lower fungi differ in their constituent polysaccharides to other fungi. The major component of microfibrils in the innermost layer of most fungi is chitin, but in P.cinnamomi and Pythium acanthium it is a branched-chain glucan polymer. This inner layer also contains protein, while the outer layer consists of a laminarin-like glucan in P.parasitica (Hunsley & Burnett, 1970). Growth at low water potential appears to have resulted in a third, innermost layer without microfibrils but containing amorphous membrane

material.

It does not appear that a thicker wall is necessarily the result of higher internal turgor pressures since the thinnest walls were observed in C.fastidium at -20 MPa, which also had the highest turgor potential (Table 2.3). The thickening of the wall may well be a feature which limits growth rate since extensibility of the wall is an important determinant of extension growth (Green et al., 1977). Differences in wall composition in osmotic mutants have been mentioned in Chapter 3.

Where other workers have examined morphological differences resulting from water potential, the effects have generally been minor in microorganisms but more drastic in higher plants. Gezelius and Norkrans (1970) found no difference, other than a slight decrease in size of mitochondria, in the ultrastructure of Debaryomyces hansenii grown in different concentrations of NaCl. Vacuoles were thought to contain lipid storage materials but the less salt tolerant species Saccharomyces cerevisiae lacked both conspicuous storage material and the membrane channels observed in D.hansenii. Cells of S.cerevisiae grown at various concentrations of KCl and sorbitol did not look different from controls except for 3 molar sorbitol when the cell were larger than normal and pleomorphic (Lillehoj & Ottolenghi, 1967). Similar studies have been carried out on the leaves of maize (Giles et al., 1974) and of sunflower (Fellows & Boyer, 1978) which have been grown under desiccating conditions of water potentials as low as -1.85 MPa and -2.6 MPa respectively. Disruption of membranes, both plasalemma and tonoplast, and the concentration of the cytoplasm were the most significant changes noted and the effects were specific to the type of tissue. Lipid droplets also became more frequent in the cytoplasm. Although osmotic potential was not investigated, cultural conditions altered slightly the tip structure of the fungus Allomyces arbuscula (Roos & Turian, 1977), such as the shape of the apical dome and mitochondria; microbodies, microtubules and lipid droplets were also most numerous when the hyphae were grown on hempseed than on a defined medium.

Membrane whorls were also seen in D.hansenii, sometimes within a vacuole, sometimes close to one. They also appeared in association with

amyloplast development when beetroot discs were washed in dilute calcium sulphate for 48 h (Hall, 1977), and in the distorted cells produced when species of Rhizobium were grown in yeast extract or casein hydrolysate at concentrations of 0.5 to 1% (Skinner et al., 1977). The latter effect could not be attributed to any single amino acid in the extracts. Thus though it can probably be concluded that osmotic stress may result in proliferation and/or damage to membranes, there is little to be gained in speculating further on possible functional relationships.

The demonstration of glycogen in P.chrysogenum and C.fastidium but not P.cinnamomi is further evidence for the role of polysaccharide in osmoregulation in the first two species. It is apparently a common storage material in fungi although it does not usually occur in the apical region (Burnett, 1976). An osmotic role has been demonstrated for glycogen in basiodiocarp development in Sphaerobolus. Hydrolysis of the glycogen to glucose decreases the osmotic potential so that water uptake occurs, the inner cup swells and inverts so ejecting the glebal mass (Burnett, 1976). Many of the mechanisms of spore liberation require high turgor pressure and it may be that glycogen to hexose or polyol interconversions play a general part in producing this. The reports of glycogen occurring in spores must apparently be treated with caution (Lendenmann & Rast, 1978). The glycogen like material in the spores of Agaricus bisporus was in fact identified as a heteroglucan or a mixture of glycans. Glycogen granules were not evident in thin sections of spores of C.fastidium (Plate 5.7A) or of P.chrysogenum (not shown).

THE EFFECT OF STEADY STATE OSMOTIC STRESS ON HYPHAL MORPHOLOGY

Phytophthora cinnamomi was grown on cellophane over media of different potentials adjusted with sucrose, Penicillium chrysogenum with KCl and Chrysosporium fastidium with glucose. Hyphae were prepared and examined as described above, and estimates of various hyphal parameters were made from 50 hyphae, usually taken from at least five colonies, from each of the potentials tested.

RESULTS

The mean values and standard deviations of the various parameters examined are shown in Tables 5.3, 5.4 and 5.5. The apical cell of C.fastidium frequently exceeded 600 µm, consequently its length could not easily be measured. Instead, the number of primary branches arising in the first 480 µm behind the apex were counted. The same procedure was adopted in the case of P.cinnamomi which is coenocytic.

In P.chrysogenum the length of the apical cell was significantly ($P=0.01$) dependent on the osmotic potential of the medium, although the only cells from adjacent potentials which differed significantly in length were from the two lowest potentials. Once again the relationship appeared to be related to the growth rate since the greatest length of cell was observed when the colony was grown on -2.5 MPa. The apical cell from -10 MPa colonies were only a third of the maximum length. The first two sub-apical compartments were also possibly slightly longer when the growth rate was higher.

The distance behind the tip at which the first branch or primary lateral arose is shown for all three species. Again this was greatest when the growth rate was greatest in P.chrysogenum and also for

P.cinnamomi. However in C.fastidium there was little difference in this parameter between -5 and -15 MPa, but at -20 MPa when fructose had been added to the medium, this distance increased by almost 50% and was significantly different (P=0.01) from the values for the other three potentials. This pattern was repeated when the length of the first primary lateral was examined, although in the this case the means were not significantly different. The length of the first branch in the other two species was probably also related to the growth rate, although this was less obvious in P.cinnamomi than P.chrysogenum.

Lowered potential decreased the number of branches arising from the apical cell of P.chrysogenum, although none of the means from adjacent potentials differed significantly (P=0.01). In this case the greatest number of branches arose at the highest potential tested, and thus agrees with the observation that hypoosmotic stress increases the density of the hyphal mat. However if the mean number of branches is considered on a unit length of hypha basis, then there is a maximum at -10 MPa. There was no significant effect (P=0.01) on the degree of branching in P.cinnamomi but there were far more branches per unit length in this species than in the other two. According to Shepherd et al. (1974) the A2 isolates of P.cinnamomi, which was used in the present experiments, produce more and longer branches than A1 isolates. Low water potential, even at -20 MPa with added fructose, decreased the degree of branching in C.fastidium although none of the means of adjacent potentials were significantly different. Colonies of this species grown at high water potentials also appeared denser which would be explained by a higher frequency of branching.

A comparison of the effect of the two different types of osmotica have so far produced a number of similarities. There was little difference in radial growth rates (Fig. 2.2), hyphal osmotic potential or fresh weight:dry weight ratios (Fig. 3.1). However, examination of the dry weight:colony radius ratios indicates that radial growth on media where KCl was the osmoticum was at the expense of dry weight production when compared to colonies grown on sugar (Table 5.6). This difference was more obvious in P.cinnamomi than P.chrysogenum. Since the fresh weight:dry weight ratios were similar, this must have been due to increased density of hyphae per unit area, that is the degree of

branching.

One of the taxonomic characteristics of P.cinnamomi is the occurrence of coralloid hyphae or frequent rounded nodules. Although this feature is difficult to quantify, qualitatively it appeared that low water potential reduced the number of nodules so that the hyphae appeared smoother and straighter than those grown at high water potential. Hyphal swellings which are also characteristic of this species when grown on malt agar, were not observed when CYA was the nutrient medium, irrespective of potential.

Considerable variation in the shape of the hyphal apex was seen in P.cinnamomi; sometimes it appeared pointed, particularly at -1 MPa, and at others rounded or even club shaped. Tip shape was also variable in C.fastidium but appeared to be fairly standard in P.chrysogenum. Some hyphal diameters are shown in Table 5.7. Those for P.cinnamomi appeared to correspond fairly closely with those obtained from the electron micrographs (Table 5.2). Actual values of mature hyphae were about 50% greater than the EM values, but the pattern with respect to potential was similar. The hyphae of C.fastidium were considerably larger when measured under the light microscope. This may have been partly due to high turgidity resulting from the glucose mountant (see Plate 5.10) or due to error in measurement because of the high refractive index of the mountant (Corry, 1976b). Also, hyphae from -5 MPa were smaller than those from other potentials which appeared to be the reverse of the trend observed in the electron microscope studies (Table 5.2).

Apical or sub-apical (dichotomous) branching as described by Trinci (1970) occurred in addition to lateral branching in all three species, but was most common in C.fastidium as indicated by the tip to first branch ratios (Tables 5.3, 5.4 & 5.5). Up to three branches were seen to arise from an apex and these were of the same diameter whereas lateral branches were considerably smaller in diameter. Branch initiation obviously caused the growth rate to decrease, since when one of a pair of dichotomous branches had itself branched, it was slightly shorter than the unbranched member of the pair. Swelling of the apex as described by Trinci (1970), to a 'hammer' shape preceded branching in this species. The tip to branch ratio was greatest and most variable in

P.chrysogenum but it did not appear to be a function of water potential.

Table 5.3. Effect of osmotic potential on the morphology of Phytophthora cinnamomi tips. Values are means and standard deviations of 50 hyphae. LSD (P=0.01) for apex to first branch was 42 µm and for length of first branch was 24 µm. Values from adjacent potentials which differed significantly are marked with a bar.

ψ_s (-MPa)	Apex to first branch (A) (µm)	Length of first branch (B) (µm)	No of branches in first 480 µm	A:B
0	188±96	57±42	4.38±1.99	4.37±3.02
0.5	192±83	56±44	4.82±2.36	4.85±3.60
1.0	173±80	81±71	5.69±2.46	3.95±3.30
2.0	175±83	43±30	5.12±1.87	5.91±5.00
2.5	150±71	57±37	4.70±2.17	3.37±2.26
3.0	138±69	55±44	5.64±2.47	3.42±2.03

Table 5.4. Effect of osmotic potential on the morphology of Penicillium chrysogenum tips. Values are means and standard deviations of 50 hyphae. LSD (P = 0.01) for number of branches per apical cell was 0.33 and for apical cell length was 36 µm. Values from adjacent potentials which differed significantly are marked with a bar.

ψ_s (-MPa)	Apical cell length (µm)	First sub-apical compartment (µm)	Second sub-apical compartment (µm)	Apex to first branch (A) (µm)	Length of first branch (B) (µm)	No of branches per apical cell	A:B
0	280±76	22±7	31±10	164±127	94±87	1.30±1.09	3.22±5.01
2.5	298±78	29±10	33±12	171±131	101±95	1.10±1.04	6.75±25.89
5.0	295±85	31±11	38±12	114±81	81±71	1.10±0.84	1.62±0.55
7.5	139±67	26±10	25±10	86±70	58±48	1.08±0.78	2.24±3.11
10.0	90±25	21±7	22±8	72±38	46±34	0.80±0.57	4.11±8.63

Table 5.5. Effect of osmotic potential on the morphology of Chrysosporium fastidium tips. Values are means and standard deviations of 50 hyphae. LSD (P=0.01) for apex to first branch was 96 µm and for the number of branches was 0.50. Values from adjacent potentials which differed significantly are marked with a bar.

ψ_s (-MPa)	Apex to first branch (A) (µm)	Length of first branch (B) (µm)	No of branches in first 480 µm	A:B
5	215±157	157±142	1.84±1.02	1.92±1.67
10	219±171	186±164	1.58±0.90	1.31±0.45
15	214±162	161±138	1.56±1.05	1.64±1.25
20	319±232	263±217	1.08±0.83	1.61±2.09

Table 5.6. A comparison of the colony dry weight to radius ratios of Penicillium chrysogenum and Phytophthora cinnamomi grown on media of osmotic potentials produced with KCl and with sugar. Data are taken from Chapter 3.

ψ_s (-MPa)	Dw : radius		ψ_s (-MPa)	Dw : radius	
	P.cinnamomi*			P.chrysogenum**	
	sucrose	KCl		glucose	KCl
0	5.46	5.06	0	9.36	8.51
0.5	3.35	3.73	2.0	10.05	8.26
1.0	3.81	1.47	4.0	10.82	7.75
1.5	2.37	0.81	6.0	9.31	6.41
2.0	1.87	0.24	8.0	6.79	6.57
2.5	1.54	0.03	10.0	4.52	5.78

* 5 days.

** 10 days.

Table 5.7. Effect of osmotic potential on hyphal diameter in Phytophthora cinnamomi and Chrysosporium fastidium. Values are means and standard deviations of 50 hyphae.

	Hyphal diameter (μm)				
ψ_s (-MPa)	P.cinnamomi		ψ_s (-MPa)	C.fastidium	
	Tip*	Hypha**		Tip*	Hypha**
0	3.1±0.6	6.5±0.8	5	5.2±0.9	7.4±1.3
0.5	3.2±0.8	6.6±0.6	10	7.5±2.0	9.5±1.4
1.0	2.9±1.0	6.4±0.6	15	7.9±1.2	9.3±1.5
2.0	3.8±0.7	6.1±0.7	20	7.7±1.6	10.0±2.2
3.0	2.9±0.7	5.5±0.6			

* Measured just behind tapered portion of apex.

** Measured ca 500 μm behind apex.

DISCUSSION

Two effects are probably reflected in these data; one is a correlation with the growth rate which was seen in the length of the apical cell in P.chrysogenum and also the distance behind the apex at which the first primary lateral arose. Secondly there may have been some morphogenetic effect due to low potential increasing the degree of branching per unit length, but again only in P.chrysogenum. In this respect C.fastidium seems well adapted to low water potential in that none of the parameters examined were greatly altered, except when fructose was added at -20 MPa resulting in an increase in length.

In contrast, in Aspergillus nidulans, the number of branches per hypha (of equivalent length) depended on the growth rate (produced with different media) and the length of the hypha (Katz et al., 1972). Another factor found to affect branching was the depth of medium. This did not affect the colony growth rate of A.nidulans but there was a significant increase in hyphal density of this species with increasing depth of medium (Trinci, 1969). In Mucor hiemalis differences in density were the result of variation in the number of branches which continue to grow once formed rather than an increased frequency of branching.

The variation in the shape of the apex observed, particularly in P.cinnamomi, was probably also the result of the alteration of the growth rate. The extension zone of the apex, that is the portion which actually extends as distinct from the growth zone which supplies that extension, is now recognised as being equivalent to the tapered region of the tip (e.g. Trinci & Halford, 1975). The length of the extension zone has been shown to be a function of the growth rate so that the tapering of the tips observed, particularly at -1 MPa, reflects the faster growth rate at this potential. However the variability of the morphological characteristics of P.cinnamomi is a well known feature of this species (Blackwell, 1949), so that statistical analysis would be necessary to determine the significance of this observation.

Prosser and Trinci (1979) have developed a model to describe hyphal growth and branching but at present it applies only to the exponential phase of growth which precedes linear growth in liquid culture. Branch initiation is determined in the model when a critical concentration of vesicles is reached in the apex or sub-apical compartment. The model results were in good agreement with experimental data. Unfortunately it was not possible to determine the effect of potential on the concentration of apical vesicles in the fungi being considered here (see previous section).

EFFECT OF OSMOTIC SHOCK ON GROWTH AND MORPHOLOGY

Phytophthora cinnamomi, Penicillium chrysogenum and Chrysosporium fastidium were grown in Petrislides on cellophane overlying agar of various potentials. Steady state hourly growth rates were determined usually by measuring tip advancement from a branch marker against a micrometer scale. Measurements were made of three hyphae from each potential, each being measured three times at 10 min intervals in P.cinnamomi, every 20 min for P.chrysogenum and 30 min for C.fastidium. In some cases, hourly rates were calculated from the daily steady state growth rates reported previously.

The colonies were treated with various types of osmotic shock in the usual way, and the time and manner in which growth resumed was noted. All observations were made at 25°C.

RESULTS

P.cinnamomi was subjected to hyperosmotic shock from 0 to -2 MPa sucrose. In one case the tip started to regrow within 1 h and the sequence of events is illustrated in Plate 5.9 A-G. In another case however, the tip did not regrow, but growth eventually resumed in the first primary lateral behind the leading hypha. This took some 5 h. In both cases, the new growth rate was close to the steady state growth rate for -2 MPa sucrose colonies.

Following hypoosmotic shock from -2MPa to 0 MPa, in one case bursting did not occur and regrowth started from the tip after about 1 h, but with a different orientation, and was followed by considerable lateral branching. In a second case, which is illustrated in Plate 5.9 H-J, there was extensive bursting. The burst tips clearly did not

recover and growth recommenced after about 3 h at branches behind the second or third lateral, that is about 100 to 200 μm behind the apex.

Clearly the effect of both treatments was fairly variable and the time taken for recovery dependent on the degree of destruction of the apex. If the apex remained intact, regrowth was much more rapid than if reorganisation of a lateral to become the leading hypha was necessary.

When P.chrysogenum was transferred from 0 to -10 MPa glucose, growth did not resume, but a shock from -2.5 MPa KCl to -10 MPa KCl resulted in regrowth after 7 h which was initiated by branching at the apex. In both cases there was an initial massive shrinkage of the colony such that the leading hyphae recoiled and curled. Hypoosmotic shock from both -10 MPa glucose and -2.5 MPa KCl to 0 MPa resulted in bursting of tips. In both treatments regrowth started within 2 h but this took place 250 μm behind the original colony margin.

Similarily regrowth did not occur on transfer of C.fastidium from -10 to -20 MPa glucose, but it did after transfer from -5 to -1.25 MPa, although only after more than 12 h. Regrowth following hypoosmotic shock of -20 to -10 MPa was also slow and took about 8 h; it occurred about 350 μm behind the margin. In all cases the new growth rate was comparable to the steady state growth rate for that potential. These results are summarised in Table 5.8.

Plate 5.9. Effect of hyperosmotic shock (A-G) and hypoosmotic shock (H-J) on Phytophthora cinnamomi. Magnification x 138, 1 scale division = 4.7 µm. Note the swollen tip prior to branching in C and the burst tips which did not regrow in H-J.

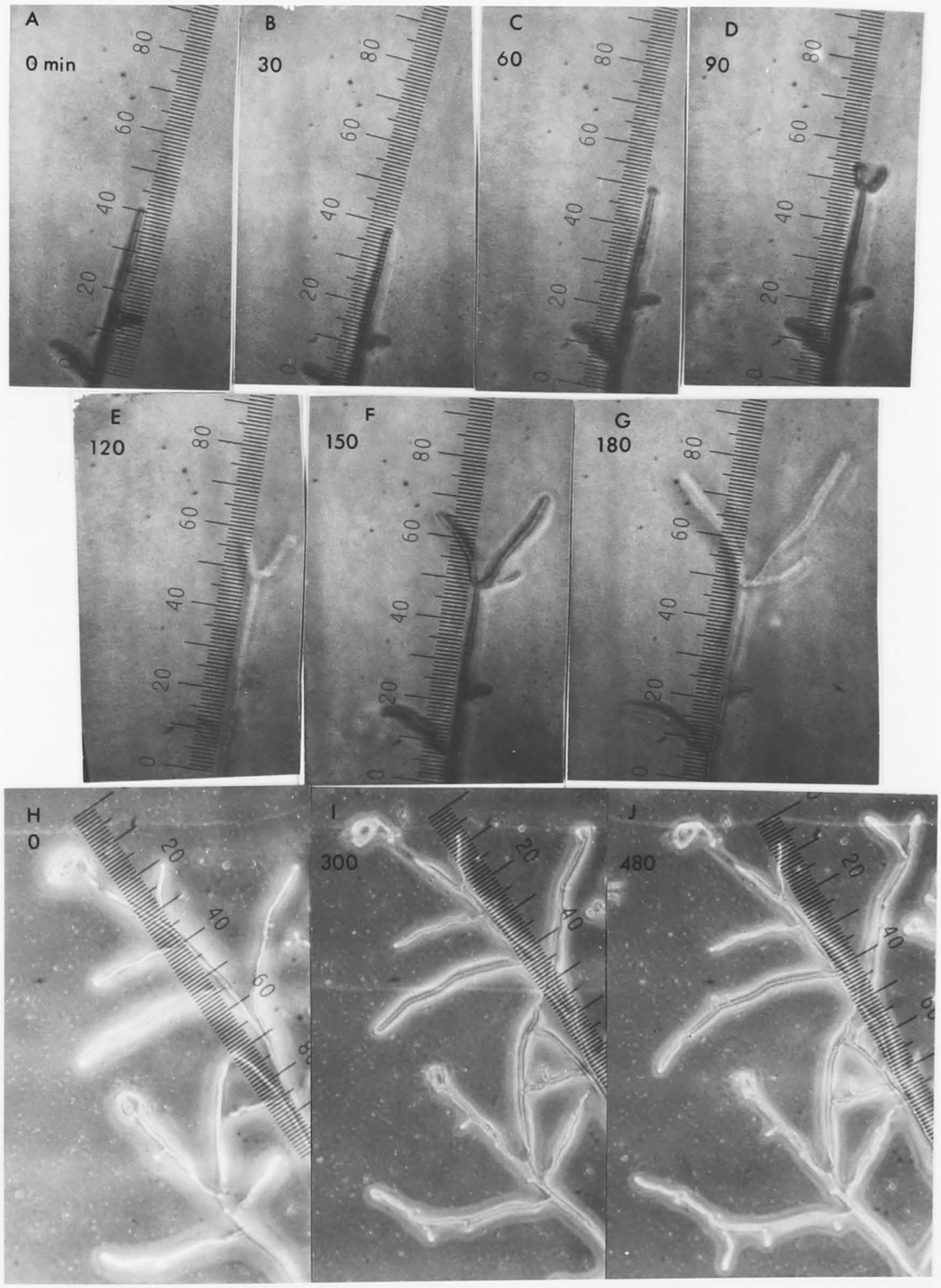

A
0 min
B
30
C
60
D
90
E
120
F
150
G
180
H
0
I
300
J
480

Table 5.8. Time taken for regrowth to occur following osmotic shock to *Phytophthora cinnamomi*, *Penicillium chrysogenum* and *Chrysosporium fastidium*.

Species	ψ(growth) (-MPa)	ψ(shock) (-MPa)	Recovery time (h)
P.cinnamomi	0	-2 sucrose	1-5
P.cinnamomi	-2 sucrose	0	1-3
P.chrysogenum	0	-10 glucose	-
P.chrysogenum	-2.5 KCl	-10 KCl	7
P.chrysogenum	-10 glucose	0	2
P.chrysogenum	-2.5 KCl	0	2
C.fastidium	-10 glucose	-20 glucose	-
C.fastidium	-5 glucose	-1.25 glucose	>12
C.fastidium	-20 glucose	-10 glucose	8

DISCUSSION

The time taken for recovery from hyperosmotic shock appears to be largely a function of the water potential difference and of the growth rate. In some cases the shock was too great for complete adaptation to occur. It has been shown (Fig. 3.12) that the glycerol content increases for 8 h following the transfer of 0 MPa P.chrysogenum colonies to -10 MPa as did the respiration rate (Fig. 4.6), which suggests that some other prerequisite for growth than synthesis of osmoregulators was affected. A shock of 2.5 MPa less than this did not prevent regrowth. Similarily a potential difference of 10 MPa inhibited regrowth in C.fastidium while 7.5 MPa did not. The latter however took longer to occur than in P.chrysogenum for a similar potential difference. The tips of Fusarium oxysporum took up to 2 h to recover and continue growing after flooding with hyperosmotic solutions (Robertson, 1958). The recovery time increased linearly with the concentration of the test solution. The growth rate of P.cinnamomi on 0 CYA was comparable to that of F.oxysporum and it also took a similar time to recover. As in my experiments just described, the first reponse of the regrowing apex of F.oxysporum was to branch. It was not possible to observe the Spitzenkörper of any hyphae in these experiments, although it is known to disappear when growth stops. Girbardt (1969) considers that its position in the apex is determined by the cell turgor and the movement of apical vesicles.

The results of hypoosmotic shock appear to depend on the degree of destruction of the tip by the bursting process. If the tip did not burst, then the recovery time was quite rapid. Tip bursting was lethal to a certain portion of the hypha, the length unable to regrow increasing with the potential difference of the shock. Working with rather smaller water potential differences, Thornton et al. (1976) have demonstrated that the amount of bursting of tips of Dendryphiella salina was a function of the sugar concentration of the flooding solution. Non-metabolizable sugars such as L-sorbose and 3-O-methyl glucose prevented bursting at high concentrations probably because they were taken up more slowly or not converted to insoluble compounds once

absorbed, so that the potential difference between medium and hypha was not excessive.

The bursting of hyphal tips is a common phenomenon and results from many treatments besides osmotic shock (see Chapter 2). It is also fairly variable within hyphae of the same treatment and probably partly a function of age. In some cases bursting may occur spontaneously for no apparent reason (Bartnicki-Garcia & Lippman, 1972), and this was also seen once or twice in C.fastidium. These authors have interpreted the bursting tendency of hyphal tips as evidence for a high lytic tendency of the wall of the apex which is normally balanced by wall synthesis. The fact that bursting was also a function of temperature as well as osmotic pressure suggested that biochemical reactions were involved. Hunsley (1973) also suggests that the thinner wall of the young apex of Phytophthora may provide a structural explanation for the bursting tendency.

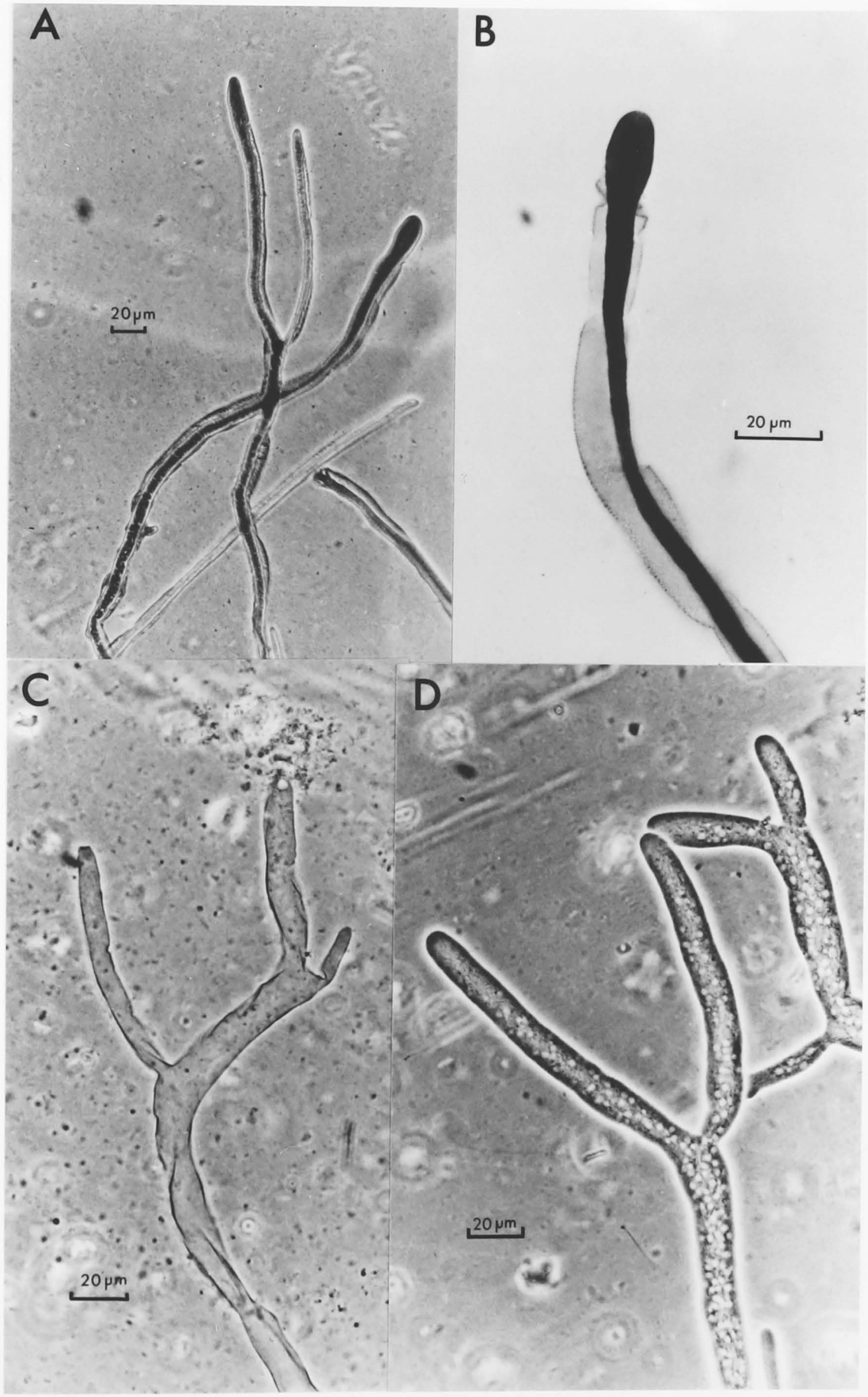
Katz and Rosenberger (1971) looked at the effect of hypoosmotic shock (distilled water) on a glucosamine-requiring mutant of Aspergillus nidulans grown on 6% w/v NaCl (-4.9 MPa) using autoradiography. Hyphal protein synthesis was not affected by the shock, but labelled glucosamine was incorporated along the whole length of the hypha instead of just at the tip. Normal tip incorporation resumed after 2 h. The fact that branching and septum formation also increased suggested that there was a relation to the mechanism initiating synthesis in specific sub-apical loci, and normal tip extension certainly appeared to be disorganised by the treatment. This seems to agree with the observation of increased branching in P.cinnamomi following hypoosmotic shock.

Finally the question of plasmolysis in fungi, about which there is considerable uncertainty, remains to be considered. Classical plasmolysis, that is the contraction of the cytoplasm from the wall, is considered to be rare in fungi, the reason suggested by Burnett (1976) being that a close connection between wall and plasmalemma exists in hyphae. However, as mentioned in Chapter 2, Thatcher (1939) did use plasmolysis (concave invaginations in the cytoplasm or convex contraction away from the tip) to determine osmotic pressure in several species. Robertson and Rizvi (1968) also described a pitting of the

cytoplasm after hyperosmotic treatment which was most obvious in older cells of Neurospora crassa as a series of concave spaces in the cytoplasm adjacent to the wall. None of these descriptions however resembles the phenomenon seen in C.fastidium mounted in lactophenol cotton blue (Plate 5.10 A & B). The force of the contraction seems to have given rise to a very obvious spiralling where the cytoplasm lies first against one wall, and then the other.

Amman's lactophenol, which contains 40% glycerol by weight, is a standard mountant for fungi although its action is not clearly understood (Dring, 1971). Dring claims that lactophenol shrinks unfixed protoplasts and the spores of some moulds disastrously, although he does not mention vegetative mycelium. It can only be assumed that a permeability change may have occurred, possibly removing glycerol from the cytoplasm with its consequent shrinkage. This drastic effect was not observed in P.chrysogenum from low potentials, although there did appear to be some pitting of the cytoplasm in older parts of the hypha. The stain and mountant used by J.I. Pitt (pers. comm.) to examine xerophilic fungi (0.1% acid fuschin in lactic acid) did not produce this effect but it did induce some bursting in C.fastidium. The use of isoosmotic mountants of sugars or electrolytes therefore seems advisable when examining fungi grown at low water potentials although it may not be possible to make accurate measurements of size (see previous two sections).

Plate 5.10. Effect of different mountants on Chrysosporium fastidium hyphae grown on -1.25 MPa glucose. A and B. Lactophenol cotton blue. C. Distilled water. D. -1.5 MPa glucose.


A
20 µm
B
20 µm
C
20 µm
D
20 µm

In other microorganisms plasmolysis appears to depend on the permeability of the solute used. Electron microscopy of Saccharomyces rouxii indicated plasmolysis of cells occurred in sucrose and sorbitol solutions only and not glycerol (Corry, 1976a). In Cyclotella cryptica, a diatom which accumulates proline, hyperosmotic solutions of all solutes including glycerol caused plasmolysis while deplasmolysis (volume recovery) was observed only in KCl and glycerol solutions (Liu & Hellebust, 1976a). Alemohammad and Knowles (1974) analysed the phenomenon in some detail in Escherichia coli. Non-penetrant solutes caused typical palsmolysis (contaction of the cytoplasm) and in addition salts caused shrinkage of the whole cell as well, while glycerol penentrated the cell and did not cause plasmolysis. Adhesion points between wall and membrane could be seen after plasmolysis by salt but not by sucrose. It therefore seems unlikely that the glycerol in the mountant is causing classical plasmolysis in C.fastidium.

6. GENERAL DISCUSSION AND CONCLUSIONS

Turgor potentials of fungal hyphae, at least at the growing margin, as determined by psychrometry (Table 2.3; Adebayo et al., 1971) are of the order of several MPa and agree well with the concentrations of osmotica considered necessary to stabilize protoplasts (Chapter 2). These values are generally higher than, for instance, those measured in algae (Cram, 1976). Algae are usually supported by their aqueous environment and so do not require mechanical strength. This difference suugests that turgor is important in maintaining rigidity and shape in filamentous fungi. There is other evidence to support this assumption. Firstly aerial hyphae of Fusarium equiseti were observed to possess higher turgor than hyphae supported by the mat (Fig. 2.2D). Secondly, Katz and Rosenberger (1971) have shown that a mutant of Aspergillus nidulans can be grown normally at high temperatures when chitin is absent, provided an osmotic stabilizer is present. Thus although there is a layer of microfibrils in the wall which presumably confers some mechanical strength, the removal of this layer does not necessarily cause disintegration of the wall. It has been pointed out in Chapter 1 that it is the filamentous growth form that allows fungi to be active in soils of low water potential. High turgor then would seem to be a prerequisite for that activity.

It has been established by various workers that linear growth of the hypha occurs only at the apex, in a region termed the extension zone which is a few microns long. This zone is supplied by the peripheral growth zone which may be of the order of several hundred microns. Behind this region the colony probably survives on a maintenance basis, or autolysis of some parts may occur allowing regrowth from well behind the colonising margin. This mode of growth must depend on directional translocation of solutes to the apex and there has been some speculation on how this is acheived. Jennings (1979) has recently reviewed the information on both transport and growth and pieced together possible mechanisms by which the two may be interrelated. Electric current flow and bulk fluid flow are two possible means by which vesicles, probably containing wall materials and enzymes, may move towards the apex. There

is undoubtedly evidence for water flow in hyphae; water droplets were seen on colonies of both P.chrysogenum and P.cinnamomi grown at high water potentials. The nutrient medium (CYA) was rich in all these experiments with a virtually unlimited energy supply at least in the sugar series. Under such conditions, presumably low hyphal osmotic potentials are generated such that some of the water taken up as a result must be excreted to avoid excessive turgor pressures. These water droplets are known to form on the tips of the hyphae of at least one fungus, Serpula lacrimans (Jennings, 1979), although no attempt was made to localize the site of transpiration in P.chrysogenum or P.cinnamomi other than to note that it occurred over the entire colony and not just at the margin. Alternatively some contractile system for moving vesicles cannot be excluded. Whatever the mechanism, there must be some signal from the extending apex to regulate the flow of vesicles, possibly originating from the Spitzenkörper.

Direct measurements of turgor, as mentioned in Chapter 2, have largely been confined to the giant algae. However these internodal cells are also cylindrical and therefore comparable in shape to the fungal hypha. Kamiya et al. (1963) have investigated the pressure-volume response of Nitella flexilis and found that extensibility of the wall, in agreement with theory, was greater in the direction of the transverse axis than the longitudinal axis in response to internal turgor pressure. Response to equal tension however was the same for both axes. The respective tensions imposed by turgor on the wall can be calculated from:

$$T \text{ (longitudinal)} = Pr/2d$$

$$T \text{ (transverse)} = Pr/d$$

where T is the tension, P the interior pressure (turgor), r the radius of the cell and d the width of the wall. If some values for these parameters from the data presented in this study are taken, for example for P.chrysogenum:

average turgor = 2.14 MPa (Table 2.3)

average cell radius = 1.5 μm (Table 5.2)

average wall thickness = 70 nm (Table 5.1)

therefore $T (l) = 22.9$ MPa

and $T (t) = 45.9$ MPa

These are rather larger values than those calculated for N.flexilis but

by extrapolation from Kamiya's data, the former would have produced an increase in length of about 1.3% and the latter an increase in width of some 2.3%.

If tensions are calculated for P.cinnamomi grown at high water potential, which has an unthickened wall, and at low potential where the wall has become excessively thickened, an interesting result is obtained:

ψ_s	= -0.5 MPa	ψ_s = -3 MPa	
P	= 0.72 MPa	P = 1.43 MPa	(Fig. 2.2B)
r	= 2.38 μm	r = 1.9 μm	(Table 5.2)
d	= 131 nm	d = 425 nm	(Table 5.1)
T (l)	= 6.5 MPa	T (l) = 3.2 MPa	
T (t)	= 13.1 MPa	T (t) = 6.4 MPa	

The net result of higher turgor, a thicker wall and a smaller cell at -3 MPa was to reduce the tension by about half from the values calculated for -0.5 MPa.

The walls of mature hyphae however are considered for practical purposes to be rigid. Apparently the microfibrils of the innermost layer of the wall generally occur at right angles to the long axis. It has been suggested that this is a mechanical adaptation to resist the greater tension exerted by internal turgor along this axis. This explanation is however by no means conclusive. Arrangement of microfibrils at the apex is random, and the microfibrils are smaller in diameter and less closely packed. These features suggest a greater susceptibility to expansion from turgor, as might be expected to account for apical extension growth. That the tips are under pressure has been well established (Chapter 2) although tip bursting is probably a chemical as well as a mechanical phenomenon. The theory (Bartnicki-Garcia & Lippman, 1972) of a delicate balance between wall lysis and wall synthesis to control this bursting tendency is an attractive one.

There was a hysteresis in the pressure-volume relation of N.flexilis conferred by the elastic properties of the wall. Dainty

(1976) has summarised the information available and emphasized the importance of the elasticity of the plant cell wall which is given by the elastic modulus (ϵ) with units of pressure:

$$dP = \epsilon dV/V$$

ϵ appears to be of the order of several tens of MPa although lower values have been calculated for higher plants; unfortunately no values exist for fungi. It is a function of turgor, decreasing to very low values as zero turgor approaches, but since it is larger than the osmotic pressure of the cell it will control the way in which the total potential of the cell changes with cell volume:

$$dV/dU = V/(\epsilon + \pi)$$

It will also control the rate of swelling or shrinkage of plant cells. It seems however that turgor is usually regulated and kept constant. Cram (1976) suggests that a negative feedback regulation of accumulatory processes would best accomplish this. There is speculation that the stretching of the plasmalemma may be the device for sensing turgor changes, or that volume or osmotic changes could be detected by certain organelles. The controlling signal may be in the form of a chemical messenger, a role for which cAMP has been suggested. There is some suggestion that levels of glucose-6-phosphate may regulate solute uptake in yeast (Becker & Betz, 1972). It has been established in the present study on filamentous fungi that turgor is regulated and that this is achieved by accumulation of solutes which respond similarily to both steady state stress and osmotic shock, but the signal to which they respond remains unknown.

It will be seen from Jennings' diagram (p. 95) that uptake of K^+ is an important feature of the translocation system he proposes. The potassium ion was certainly the predominant cation in these fungi and the fact that it was accumulated as the potential decreased at least in C.fastidium suggests that it may well be important in osmoregulation. If the view of Aiking et al. (1977) that K^+ is central to ATP metabolism is accepted then this result is not surprising. What is difficult to explain is the apparently inhibitory effect of K^+ on at least one general metabolic enzyme. An estimated KCl concentration (osmotic potential) in C.fastidium of say -1.5 MPa grown on -20 MPa glucose/fructose (Table 3.14E) would appear to inhibit the NADP isocitrate dehydrogenase activity to 10% of its maximum value (Table 4.9) while P.chrysogenum

growing on an osmoticum of KCl may have had an internal K^+ concentration of up to -9 MPa (Table 3.14D) which would have inhibited activity of this enzyme to about 6% of the maximum (Table 4.9). That this inhibition cannot be relieved significantly either by the presence of glycerol or by increasing the substrate concentration leads one to suppose that some compartmentalisation of ions may exist after all. Whether it is the flux of ions (potassium or associated protons, or possibly in some marine species, sodium) or the final internal level which is important is uncertain. Jennings suggests that the flux of K^+ across the membrane by pumps some distance from the apex and its flow to the apex essential to growth. One might speculate further that increased K^+ resulting from increased pumping (more ATP needed) at low potential coincidentally inhibits enzyme activity and so reduces the growth rate.

The potassium content of soils, expressed as K_2O ranges from 0.05 to 3.5% for mineral soils, but the proportion of this which is soluble and exchangeable is usually relatively small. Bolt et al. (1976) give values for a number of different soils ranging from 0.3% to 6.9% of the total exchangeable cations which in turn ranged from 8.8 to 60.7 mequiv/100 g soil. However such amounts are usually sufficient for plant growth, except under conditions of intensive agriculture or on sandy soils where it must be applied as a fertilizer. K^+ is also widely distributed in plant material and not tightly bound so that the parasitic or saprophytic habit is likely to provide sufficient of this ion. Competition for potassium between plants and microorganisms presumably exists although this is difficult to demonstrate. It would be interesting to establish the relative contributions of K^+ and organic solutes to osmoregulation under natural conditions. The other ions investigated do not appear to be significant in osmoregulation, and in any case are unlikely to be limiting. The availability of phosphate is more likely to be critical and should be considered.

The principal osmoregulatory compounds in the three species studied were the organic compounds glycerol and proline. Their synthesis will depend on the availability of carbon or nitrogen compounds in the substrate, as of course will all aspects of fungal survival and growth. The main source of carbon in the soil is the cell walls of plants, which

consist of complex polysaccharides which must first be hydrolysed to their constituent sugars. The principal nitrogen sources are in the form of ammonia or nitrate, although there may also be requirement for organic nitrogen in the form of amino acids. It is usually the ratio of carbon to nitrogen available to the microoganism which is considered to be of importance. C:N ratios have not been obtained in this study but Griffin (1972) states that they are of the order of 10 or 12:1 for most fungi and bacteria, but much larger for green plants and higher still in wood. The effect of microorganisms is to alter the C:N ratio of the surrounding environment eventually to a value close to their own. The C:N ratio of Czapek-Dox agar is about 40:1 and the carbohydrate to total nitrogen ratio of yeast extract is 1.6. The C:N ratio will of course become very large with the use of glucose to adjust the potential, but will be unaffected by a KCl osmoticum. It is therefore the availability of nitrogen which is most likely to be the limiting factor for both growth and osmoregulation at least in the lower fungi. Whether alteration of the C:N ratio would also alter either the type or amount of internal osmoticum produced requires investigation if survival and colonization of natural substrates is being considered.

The work on respiration was an attempt to establish the energy demands imposed by the water status of the environment. The simplest assumption is that the necessity to synthesize osmoregulatory compounds internally and prevent their leakage, would increase the need for energy per unit growth. Wilson and Griffin (1975b) indirectly established this to be the case for four fungal species to varying degrees. However they compared two potentials only for each species and used a KCl osmoticum and not sugar. One of their experimental species was P.cinnamomi where potentials of -0.1 and -4.1 MPa were compared. The result obtained that the rate of respiration per unit increase in area was ten times greater at the lower potential only partially agrees with the results presented here when the specific rate was considered (Table 4.7). However their data were extracted from a model of respiration rate proportional to the radius only which was not found to be applicable here at least in the case of P.chrysogenum. The specific rate on KCl may have increased at low potential in P.cinnamomi, but in P.chrysogenum it appeared to be more closely related to the growth rate with an optimum at -2.5 MPa. This suggests that osmoregulation may be rather less efficient in the

former than the latter species.

Catabolite repression of respiration at low potentials produced with sugar seems to have been the principal feature rather than enhanced respiration although radial growth rates were similar on KCl and sugar osmotica (e.g. Fig. 2.2). The results obtained with NADP isocitrate dehydrogenase suggest that catabolite repression by glucose is largely overcome in vitro by the presence of glycerol at least in C.fastidium and P.chrysogenum. The ability of proline to fulfil this function remains untested, although glucose was by no means as inhibitory to P.cinnamomi as to the other two species.

The availability of high energy bonds, principally in ATP, must ultimately control the performance of an organism at low water potential. The only information on this commodity in relation to osmoregulation appears to be that of Gustafsson (1979), who found increased ATP levels in Debaryomyces hansenii grown at high NaCl concentrations. More detailed information on ATP, ADP and cAMP concentrations would be useful in this respect. That the pentose phosphate pathway (which produces less ATP than the TCA cycle or respiratory chain phosphorylation) probably becomes active at low potential suggests in fact that less ATP would be available. Branch points in the oxidative sequence may also exert some control; phosphofructokinase particularly has been mentioned. The complex enzyme regulating process known as catabolite repression is another example, and the ability to overcome this may be essential for obligate osmophiles. Enzyme repression by K^+ may be the consequence of this mode of existence as discussed above. That much energy generated does not seem to be used up in the synthetic processes of growth now seems established (Forrest, 1970; Lagunas, 1976; Table 4.4). More detailed partitioning of the energy requirement is needed.

The relationship of structure to function is important in an understanding of life in extreme environments. Some aspects of the particular shape of fungi have been discussed above. More detailed investigations on ultrstructural modifications by water potential were less successful. Of particular value would be information on size and numbers of vesicles in the region of the apex and how this is affected

by water potential. Such a study however would be a major project in itself.

Attempts by various workers to relate membrane or wall structure to osmotic adaptation have been discussed, but caution must be applied in attaching causal relationships to differences in wall composition and structure. It has already been mentioned that age and cultural conditions are important determinants of morphology. Bartnicki-Garcia and Nickerson (1962) for instance have demonstrated considerable quantitative differences in the walls of the yeast and filamentous forms of Mucor rouxii but found it difficult to relate these to morphological features. It might be tempting to speculate on the interconversion between these two growth forms. Much effort has been devoted to the biochemical aspects of the transition, and levels of proteins binding cAMP are probably involved (Forte & Orlowski, 1980), but so far as is known the phenomenon has not been investigated as a function of water potential. The suggestion has been made that cAMP causes changes in the cell wall-membrane complex of Neurospora crassa which is the main determinant of its morphology (Mishra, 1976). Measurements of cAMP levels at different water potentials might prove interesting in this respect.

In terms of microscopic features, C.fastidium again appears well adapted to its environment, with hypoosmotic stress producing more obvious changes than hyperosmotic stress. P.cinnamomi is such a variable organism, even when not stressed, that effects attributable to water potential are not easily detected. P.chrysogenum falls somewhere between the two in this respect and certain features were related to the growth rate while low water potential probably increased the degree of branching per unit length.

In summary, a number of aspects of the physiological responses of fungi to low water potential have been examined: some questions have been answered but others have been raised. The answer to the original question posed concerning the nature of xerophily appears to be that the obligate xerophile and the facultative species were not radically different in their physiology, with the exception of the inability of C.fastidium to tolerate high salt concentrations. It was

the non-xerophytic species which exhibited the most noticeable differences, at least in the parameters examined in this study; the type of osmoregulator, respiration and the response of the TCA enzyme and effect of potential on wall structure can be mentioned. Throughout it must be borne in mind that the conditions imposed were highly artificial. Although studies in the natural environment, particularly soils, are notoriously difficult, it is important now to try to relate some of these findings to more natural conditions.

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APPENDIX 1

LIST OF SPECIES NAMES WITH AUTHORS MENTIONED IN THE TEXT

FUNGI

Agaricus bisporus (J.E. Lange) Singer
Allomyces arbuscula Butler
Aphanomyces euteiches Drechsler
Aspergillus flavus Link
Aspergillus nidulans (Eidam) G. Winter
Aspergillus niger van Tiegham
Aspergillus restrictus G. Smith
Aspergillus wentii Wehmer
Candida utilis (Henneberg) nov. comb.
Chrysosporium fastidium Pitt
Claviceps purpurea (E.M. Fries) L.R. Tulasne
Debaryomyces hansenii (Zopf) Lodder & Kreger-van Rij
Dendryphiella salina Nicot & Pugh
Eurotium amstelodami Mangin
Fusarium equiseti (Corda) P.A. Saccardo
Fusarium moniliforme Sheldon
Fusarium oxysporum (von Schlechtendal) f. sp. *gladioli* (Massey) Snyder & Hansen
Geotrichum candidum Link ex Persoon
Lulworthia medusa (Ellis & Everhart) Cribb & Cribb
Moniella tomentosa (Van Beyma) Stolk apud Stolk & Dakin
Mucor hiemalis Wehmer
Mucor rouxii (Calmette) Wehmer
Neurospora crassa Shear & Dodge
Ophiobolus cariceti (Berkeley & Broome) P.A. Saccardo
Penicillium canescens Sopp
Penicillium chrysogenum Thom
Penicillium notatum Westling
Phellinus noxius (Corner) G.H. Cunningham
Phycomyces blakesleeanus Burgeff
Phytophthora cinnamomi Rands
Phytophthora parasitica Dastur

Pichia miso Mogi
Piricularia oryzae Cavara
Puccinia graminis Persoon f. sp. tritici Eriksson & E. Henning
Pyrenochaeta terrestris (Hansen) Gorenz, J.C. Walker & Larson
Pythium acanthium Drechsler
Pythium debaryanum Hesse
Pythium ultimum Trow
Rhizoctonia solani J. Kuhn
Saccharomyces carlsbergensis Hansen
Saccharomyces cerevisiae Hansen
Saccharomyces oviformis Osterwalder
Saccharomyces rouxii Boutroux
Sclerotinia sclerotiorum (Libert) de Bary
Serpula lacrimans Persoon ex S.F. Gray
Thraustochytrium roseum Goldstein
Torulopsis halonitratophila Onishi
Verticillium albo-atrum Reinke & Berthold
Verticillium dahliae Klebahn
Xeromyces bisporus Fraser

ALGAE

Chlorella emersonii = Chlorella vulgaris Emerson
Chlorella pyrenoidosa Emerson
Cyclotella cryptica (Reimann) Lewin & Guillard
Dunaliella parva Lerche
Dunaliella tertiolecta Butcher
Dunaliella viridis Teodoresco
Griffithsia monilis Harvey
Monochrysis lutheri Droop
Nitella flexilis (Linnaeus) C.A. Agardh
Ochromonas malhemensis Pringsheim
Platymonas subcordiformis (Wille) Hazen
Stichococcus bacillaris Naegeli

HIGHER PLANTS

Lemna minor Linnaeus
Plantago maritima Linnaeus
Suaeda maritima (Linnaeus) Dumwort
Triglochin maritima Linnaeus

BACTERIA†

Escherichia coli
Halobacterium salinarium
Pseudomonas fluorescens
Salmonella orianenburg
Staphylococcus aureus
Vibrio alginolyticus

† It is not conventional to give authors to bacterial species.

APPENDIX 2

OSMOTIC POTENTIALS OF MANNITOL SOLUTIONS

Osmotic potentials of mannitol solutions were determined, as described in Chapter 2, by thermocouple psychrometry. Values are the means of five determinations. The solubility of mannitol is 17.7% at 25°C.

Concentration (molal)	ψ_π (-MPa)
0.1	0.49
0.2	0.77
0.3	1.06
0.4	1.33
0.5	1.61
0.6	1.89
0.7	2.10
0.8	2.39
0.9	2.66
1.0	2.89
1.1	3.14

Linear regression

$$\psi_\pi = (\text{molality}/0.38) + 0.97 \quad (-\text{MPa})$$